THE DISTRIBUTION AND BIOLOGY OF AMOEBAE IN FRESHWATER IN SOUTH AUSTRALIA

WITH SPECIAL REFERENCE TO THE EPIDEMIOLOGY AND CONTROL OF AMOEBIC MENINGOENCEPHALITIS

by

Bret S. Robinson, B.Sc.(Hons).

Department of Zoology, University of Adelaide,
South Australia.

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CONTENTS

SUMMARY

A study of the distribution of free-living amoebae in freshwater has been carried out by regular sampling of water sources and public water supplies in South Australia. Most of the genera recognized in a recent 'Illustrated Key' were identified, many of them recorded for the first time from Australia. Particular attention was given to identification of *Naegleria fowleri*, the amoeba responsible for thirteen fatal cases of primary amoebic meningoencephalitis in South Australia, and of nonpathogenic *Naegleria* species. Descriptions of two new species of Vahlkampfiid amoebae, which must be distinguished carefully from *Naegleria* species, are presented.

The upper limit of temperature tolerance was determined for over 400 *Naegleria* isolates from water samples. For *Naegleria* species isolated from three South Australian reservoirs, temperature tolerance correlated significantly with seasonal changes in water temperature. The distribution of 'high temperature' strains, including *N. fowleri*, corresponded with the seasonal and geographic distribution of the recorded infections.

A mathematical description of the influence of temperature on growth rates was derived from growth experiments with *N. fowleri* and *N. gruberi*. The seasonal changes in abundance inferred from the correlation with water temperature could be explained partly in terms of the lower limits of temperature tolerance and temperature coefficients (Q_{10}) of growth calculated from the growth equations. The influence of temperature on other physiological processes is also discussed.

Acanthamoeba species, which may cause infections of varying severity, including fatal meningoencephalitis, were also identified. Temperature tolerance of 245 isolates was relatively uniform, and no seasonal trend in their isolation could be demonstrated.

The effectiveness of chlorine in control of *Naegleria* species was studied in a number of disinfection experiments. The effect of chlorine on *N. fowleri* cysts was influenced by chemical and physical variables in a manner predicted from a knowledge of the chemistry of chlorine in water. Under most conditions encountered in practice, survival of *N. fowleri* cysts was reduced to below one per cent by a chlorine dose sufficient to provide 0.5 $mg.l^{-1}$ free chlorine after 30 minutes contact.

Initial kinetics of the action of chlorine did not fit the 'pseudo-first order' model of disinfection often reported for disinfection of bacteria and viruses. The survival curve suggested a finite 'penetration time', shorter for *N. fowleri* than for *N. gruberi* cysts, which were less susceptible to chlorine.

While trophozoites of *Acanthamoeba* were very susceptible, cysts of *A. castellanii* and *A. polyphaga* were extremely resistant to chlorine.

A comparison of the frequency of isolation of amoebae before and after chlorination at a number of water sources confirmed the effectiveness of chlorine against *Naegleria* species and the longer survival times of *Acanthamoeba* cysts. Possible sources of the occasional contamination of reticulated water by *Naegleria gruberi* and other nonpathogenic strains are discussed.

DECLARATION

This thesis contains no material which has been presented for the award of any other degree or diploma in any university. To the best of my knowledge and belief, it contains no material previously published or written by another person, except where due reference is made in the text of the thesis.

Bret Robinson.

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1. INTRODUCTION

1.1 PRIMARY AMOEBIC MENINGOENCEPHALITIS

Acute human infection by amoebae other than those considered obligate parasites was first reported in South Australia (Fowler and Carter, 1965) and Florida, U.S.A. (Butt, 1966).

The infection, popularly known as 'amoebic meningitis', is more correctly a meningoencephalitis, and has been fatal in sixteen of seventeen recognized cases in Australia. Meningoencephalitis is the primary infection, which distinguishes it from infection of the central nervous system by *Entamoeba histolytica* (secondary to infection of the gastrointestinal tract) which has been observed occasionally (Lombardo *et al.*, 1964). Hence the name primary amoebic meningoencephalitis (Butt, 1966), often abbreviated to PAM, is widely used.

The South Australian infections were originally attributed to *Acanthamoeba* by Fowler and Carter (1965), who were aware of a growing literature concerning *Acanthamoeba* isolated from mammalian cell cultures (Jahnes *et al.*, 1957; Culbertson *et al.*, 1958). Cytopathic effects observed in the cultures had led to attempts to infect laboratory animals by various routes. Infection of the central nervous system following intranasal inoculation was one of the important observations (Culbertson *et al.*, 1959; Culbertson, 1961).

Six human cases described in detail by Carter (1968) did not show the extensive pulmonary involvement which was demonstrated in many of the experimental infections. A more detailed morphological study followed cultivation of amoebae from one of the cases, and the amoeba-flagellate transformation characteristic of *Naegleria* was observed (Carter, 1968). Callicott (1968) also isolated *Naegleria* from a fatal case in Virginia, U.S.A. and suggested that seven cases diagnosed in retrospect from pathology material (Callicott *et al.*, 1968) might all be attributed to *Naegleria*. Cases of amoebic meningo-

encephalitis have now been described from Australia, New Zealand, Great Britain, Belgium, Czechoslovakia, several areas of the U.S.A. and parts of Africa. *Naegleria* has been isolated from many infections and is believed to have been responsible for most cases. The few confirmed human infections by *Acanthamoeba* are discussed in Section 1.3.

Pathogenic strains of *Naegleria* were given specific rank as *Naegleria fowleri* by Carter (1970), distinguishing them from the previously recognized species, *N. gruberi*.

Naegleria species produce cysts under conditions which have not been defined, although low nutrient levels certainly stimulate encystment. Amoebae of this genus are widely distributed in freshwater (Page, 1967a) and can be isolated from soil (Singh, 1952), though they may be less common in that environment than *Acanthamoeba* species (Page, 1967b). Although *Naegleria* species have been isolated from air-borne dust (Jamieson, 1975), cysts of *Naegleria fowleri* appear to be less resistant to desiccation than those of some nonpathogenic strains, or those of *Acanthamoeba* species (Chang, 1978).

Experiments with laboratory animals suggest that cysts are not infective, and air-borne cysts are considered an unlikely source of human infection (Carter, 1970). From histological observations on fatal cases and by analogy with infection of laboratory animals, intranasal infection is considered to be the route of entry by *Naegleria fowleri*, and primary amoebic meningoencephalitis is presumed to be invariably waterborne.

Circumstances surrounding the reported cases of the disease differ between the countries where it has occurred, and are discussed in detail in Section 4.1. The link between the groups of cases appears to be swimming (or at least 'recreation') in freshwater which is unusually warm. Although amoebic meningoencephalitis is very uncommon,

no other link between victims, suggestive of peculiar susceptibility to the infection, has been established. It seems that infection by *Naegleria fowleri* has a very low probability and that the circumstances in which infection may occur are likely to involve healthy, active persons.

The epidemiology of primary amoebic meningoencephalitis in South Australia is less simple than in some other countries. The thirteen cases occurred over a period of seventeen years in three well-separated country towns: Pt Augusta, Pt Pirie and Kadina (Table 1.1 and Figure 1.1). There was some history of swimming in only seven of eleven cases for which information was available, and one of those had swum only in seawater (Carter, 1970). Although *Acanthamoeba* species are widely distributed in the sea (Davis *et al.*, 1978), *Naegleria* species are intolerant of high salinities and have not been isolated from seawater (Page, 1976).

TABLE 1.1

AMOEBIC MENINGOENCEPHALITIS: AUSTRALIAN CASES

South Australia 13; Western Australia 3*; Queensland 1

Pt Augusta (8): 1961, 1963, 1965 (2), 1970, 1972 (2)

Pt Pirie (3): 1955, 1971 (2)

Kadina area (2): 1965, 1969

9/13 cases under 11 years old; 7 male, 6 female

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* includes one infection by *Acanthamoeba* (see Section 1.3)

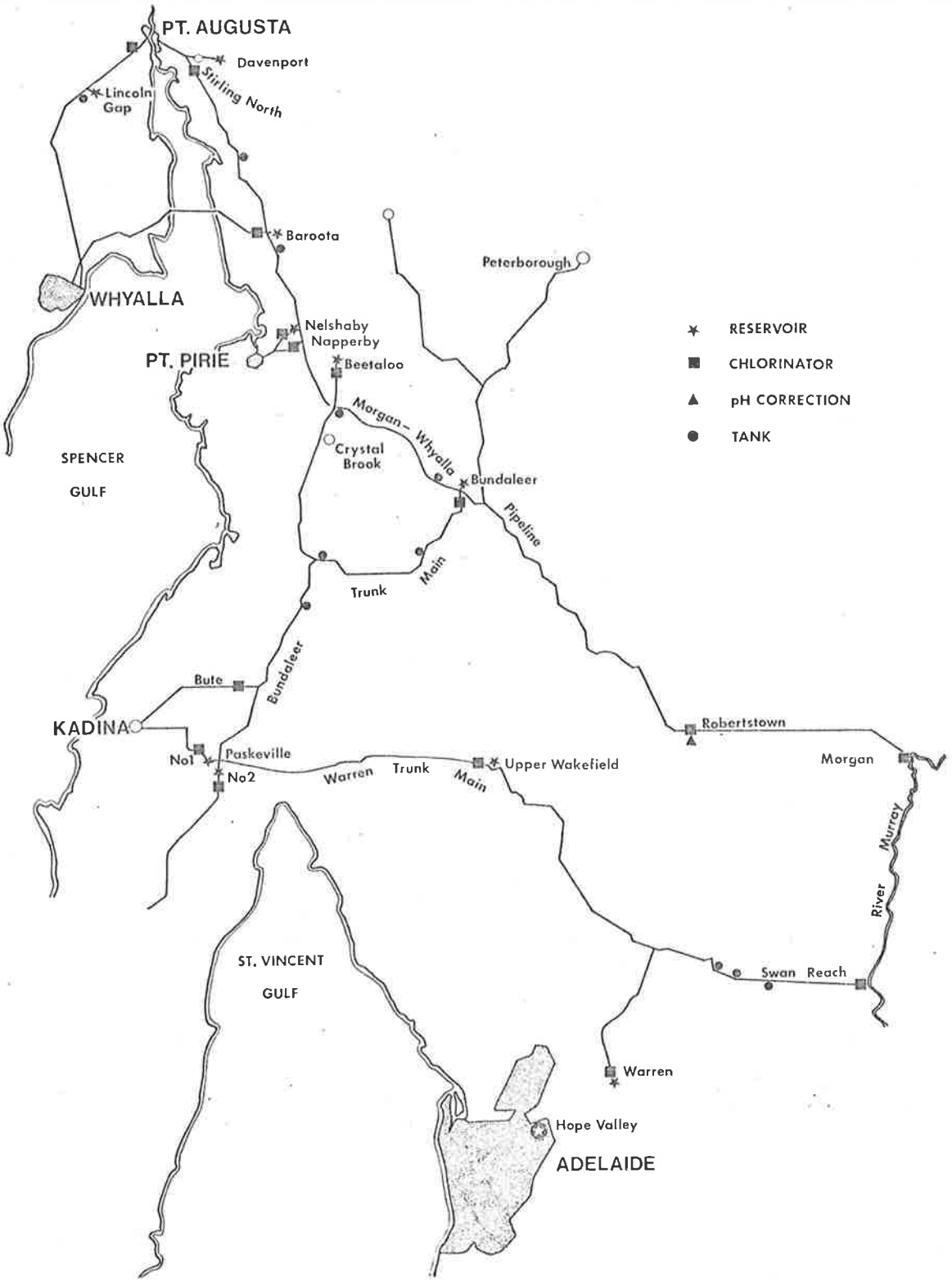


FIGURE 1.1 WATER SUPPLY TO NORTHERN SOUTH AUSTRALIA.

The State Water Laboratories of the Engineering and Water Supply Department became involved in the study of amoebic meningo-encephalitis in South Australia when evidence of a link between one case and the domestic water supply was published (Anderson and Jamieson, 1972a). The absence of a history of swimming in some earlier cases also suggested that the public water supply was a possible source of infective organisms.

1.2 WATER SUPPLY TO NORTHERN SOUTH AUSTRALIA

The sources of water supplied to Pt. Augusta, Pt. Pirie and Kadina are illustrated in Figure 1.1, with details of storages and points of disinfection.

Most of the water required is pumped from the River Murray at Morgan and Swan Reach. Some water is supplied directly from the trunk mains, but there is also long-term storage in reservoirs, some of which have a local catchment. Short-term storage in large concrete tanks such as those at Lincoln Gap facilitates supply during fluctuations in demand. The significance of reservoirs and tanks as points of contamination is discussed in Section 5.5.

The Morgan-Whyalla Pipeline supplies most of the water required by Pt. Augusta, Pt. Pirie, Whyalla and a number of smaller population centres. The original pipeline, completed in 1944, was constructed above ground to Pt. Augusta and south to Whyalla. In 1967, a second pipeline, mostly below ground, was completed alongside the first to Baroota and under Spencer Gulf. The pipes have a combined capacity of 2400 l/sec. at Morgan and deliver effectively the same water, since they are cross-linked at intervals.

Until 1972, water was chlorinated at Morgan for two periods of eight hours each week, largely for 'slime control' in the pipeline. Stations providing continuous disinfection have since been constructed

at most of the water sources, as indicated on Figure 1.1.

The immediate sources of water for Pt. Pirie are the Morgan-Whyalla Pipeline at Napperby, and Nelshaby Reservoir. The reservoir has a small local catchment, mainly providing storage for River Murray water from the pipeline. After disinfection, Nelshaby water mixes with water drawn directly from the pipeline (rechlorinated at Napperby), to supply Pt. Pirie.

Pt. Augusta is usually supplied with water from the Morgan-Whyalla Pipeline, rechlorinated at Stirling North. If supply is restricted (for example, by repair work on the pipeline), water from the open concrete-lined storage at Lincoln Gap is used and a chlorinator at Pt. Augusta West provides temporary disinfection. Water from local springs in the South Flinders Ranges, collected in Woolundunga and Davenport Reservoirs, was the original supply to Pt. Augusta, but is now used only for the small community of Stirling North.

Whyalla is usually supplied with water from the River Murray and from the South Flinders catchment of Baroota Creek, through the Morgan-Whyalla No. 2 Pipeline under Spencer Gulf. Water can also be supplied through the original pipeline from Lincoln Gap Tanks or Lincoln Gap Storage.

Water supply to Kadina is more complex. The immediate sources are a branch of the Bundaleer Trunk Main with rechlorination at Bute, and the Paskeville No. 1 Reservoir. The Bundaleer Trunk Main usually carries water from the Morgan-Whyalla Pipeline, but water from the catchment of Bundaleer Reservoir, which includes a diversion from the Broughton River, is sometimes available.

Paskeville No. 1 Reservoir is filled from the Warren Trunk Main, which carries water from the River Murray at Swan Reach or from Warren Reservoir near Adelaide, and from the Bundaleer Trunk Main.

Paskeville No. 2 Reservoir, which supplies water to most of southern Yorke Peninsula, is filled from the same sources. Neither reservoir has a local catchment.

The detail on water supply in areas where infection by *Naegleria* is a risk is provided to illustrate how diverse the likely sources of contamination by freeliving soil/freshwater organisms are. It also provides a basis for the field sampling reported in Section 5.5, where the relative importance of sources of contamination and the effectiveness of disinfection are assessed.

1.3 HUMAN INFECTION BY *ACANTHAMOEBA*

Most of the cases of amoebic meningoencephalitis in the world have now been attributed to *Naegleria fowleri*, either by isolation and identification of the amoebae or from the short clinical course typical of the 'reference' cases. A few cases have now been attributed with certainty to *Acanthamoeba*. The criteria used have included serological tests using complement fixation (Kenney, 1971) and fluorescent antibody technique (Jager and Stamm, 1972; Willaert *et al.*, 1978). Features of *Acanthamoeba* meningoencephalitis recognizable from experimental infections have helped in some cases: presence of cysts in tissue (Jager and Stamm, 1972; Robert and Rorke, 1973; Bhagwandeen *et al.*, 1975), protracted course of the disease (Robert and Rorke, 1973, Ringsted *et al.*, 1976) and involvement of other organs in the infection (Ringsted *et al.*, 1976, Bhagwandeen *et al.*, 1975).

A case described by Sotelo-Avila *et al.* (1974) illustrated the difficulty of identifying the amoebae responsible from histology alone. The pathology material was submitted to three independent medical scientists with experience of amoebic meningoencephalitis. Two of them identified the amoebae seen in sections as *Acanthamoeba*, the other believed that they were *Naegleria* (Sotelo-Avila *et al.*, 1975).

Amoebae have not been cultivated from any of the meningoencephalitis cases attributed to *Acanthamoeba*. Contact with water as a likely source of infection has been recorded for only one case. There is little evidence of infection by the intranasal route, as has been common to the cases attributed to *Naegleria*. In two cases there was evidence of entry through a skin lesion on the face (Ringsted *et al.*, 1976) or abdomen (Bhagwandeen *et al.*, 1975) followed by haematogenous spread. It is possible that in most cases the immediate origin of the infecting *Acanthamoeba* was soil.

The virulent pathogen *Acanthamoeba culbertsoni*, used in most of the experimental studies (Culbertson, 1971), has been specifically identified in only one human infection (Willaert *et al.*, 1977). In some experimental studies, *Acanthamoeba* strains of lower virulence to laboratory animals have been isolated from water or soil (Culbertson *et al.*, 1966; Singh and Das, 1970). These isolates were assigned to several species, and were usually 'noninvasive'; that is, they required a preexisting lesion for successful infection.

Acanthamoeba strains have been cultivated from three human infections less serious than meningoencephalitis (Visvesvara *et al.*, 1975; Nagington *et al.*, 1974). The three cases were eye ulcerations, two attributed to *A. polyphaga* and one to *A. castellanii*. The protracted course of two infections resulted in loss of sight despite corneal grafts. The third case was recognized within a few days and treated successfully (Visvesvara *et al.*, 1975). Two of the cases were farmers who had suffered minor injury to the eye during outdoor work. This suggests a soil origin for the infecting amoebae.

Acanthamoeba has been included in the experimental and field studies presented in this thesis because of the ability of *A. culbertsoni* to infect by the same route as *Naegleria fowleri*, the widespread occurrence of *Acanthamoeba* in fresh water and soil, and the

range of *Acanthamoeba* strains which may have some virulence for man. A fatal meningoencephalitis in a Western Australian child in 1974, recently attributed to *Acanthamoeba* (Carter, pers. comm., 1980), is the only infection by amoebae of this genus which has been recognized in Australia.

1.4 OBJECTIVES

The study presented in this thesis will attempt to relate the distribution and biology of amoebae, particularly *Naegleria* and *Acanthamoeba* species, to the epidemiology of amoebic meningoencephalitis. The following questions will be addressed: What kinds of amoebae are present in water which is to be used for domestic supply in South Australia, and what is their public health significance? How does their seasonal and geographical distribution relate to the recorded incidence of primary amoebic meningoencephalitis in South Australia? Can they be controlled by conventional methods for disinfection of water?

2. MATERIALS AND METHODS

2.1 ORIGIN OF AMOEBA CULTURES

Several strains of amoebae isolated by other laboratories were used in temperature tolerance and disinfection experiments. These included pathogenic *Naegleria* strains isolated from human infections. Details of the origin of each strain and the immediate source of the culture are given below.

Abbreviations -

ACH - Adelaide Children's Hospital (Immunology Dept.)

ATCC - American Type Culture Collection

CCAP - Culture Centre of Algae and Protozoa, Institute of Terrestrial Ecology, Cambridge, England

IMVS - Institute of Medical and Veterinary Science (Amoebic Research Unit)

SWL - State Water Laboratories, Engineering and Water Supply Dept., South Australia.

Strain *Naegleria gruberi* CCAP 1518/1c

Origin fresh water, Britain

(IMVS)

Naegleria fowleri NORTHCOTT (ATCC 30462)

PAM case, Pt. Augusta, 1971

(ACH)

N. fowleri MORGAN (ATCC 30465)

PAM case, Pt. Augusta, 1972

(IMVS)

Strain	*N. fowleri* DAMIANO
Origin	PAM case, Merridin, W.A., 1980 isolated by the author from cultures prepared at Princess Margaret Childrens Hosp., Perth, W.A.
	N. fowleri NHI (ATCC 30475) PAM case, New Zealand, 1973 (IMVS)
	Acanthamoeba castellanii NEFF (CCAP 1501/1) soil, California, U.S.A. (IMVS)
	A. polyphaga SWL FF-1 sewage effluent, isolated in mammalian cell culture by Fairfield Infectious Diseases Hospital, Melbourne, Victoria; sent to SWL for identification.

2.2 MEDIA AND CULTIVATION

Maintenance of reference strains of amoebae, isolation of amoebae from field samples and preparation of amoebae for experimental work were all carried out on solid, non-nutrient media spread with bacteria. Non-nutrient agar was prepared by dissolving Difco Bacto-Agar (1.5% w/v) in reverse-osmosis deionized water, straining and autoclaving in approximately 300 ml volumes.

For most cultures, 90 mm disposable plastic petri dishes (Disposable Products) containing 14 ml of non-nutrient agar were used. However, temperature tolerance of individual amoeba strains was measured using disposable 36 mm petri dishes containing 3 ml of agar. All such culture plates were poured in a laminar flow cabinet (Gelman-Clemco) to prevent air-borne contamination.

The amoebae which were of greatest importance in this study feed on a range of gram-negative bacteria. It was convenient to cultivate amoebae from field samples, and to sub-culture all *Naegleria* and *Acanthamoeba* strains and most other reference strains, using heat-killed *Escherichia coli*. *E. coli* from an over-night culture on plate count agar was suspended in quarter-strength Ringer solution and incubated in 2 ml aliquots at 60°C for 60 minutes. Non-nutrient agar plates were spread with a drop of this suspension from a Pasteur pipette, using a sterile glass rod. To check the sterility of the media and the effectiveness of heat-killing, sample plates from each batch were incubated at 28°C for 48 hours. 'Positive' quality control - tests of the ability of the media to support growth of a *Naegleria* subculture - was also carried out.

Suspensions of cysts of *Naegleria* or *Acanthamoeba* for disinfection experiments (Section 5) were prepared from such plate cultures. 48 hours after inoculation, plates were respread with *E. coli* (a few additional drops) to increase the yield of cysts. After a period of incubation, usually 4 days, during which multiplication and encystment occurred, cysts were harvested in 0.05% Triton X-100, using the glass rod to suspend them from the agar. The Triton X-100 ensured that the cyst suspension was free of viable trophozoites, which are sensitive to surfactants (Preston and O'Dell, 1973). Cysts were washed by 3 or 4 consecutive centrifugations in a bench centrifuge, resuspended each time in quarter-strength Ringer solution. Before use in an experiment, the cysts were counted microscopically, and the absence of viable trophozoites confirmed, using a haemocytometer cell.

Some other Vahlkampfiid strains grew poorly and encysted after a few generations, if subcultured on heat-killed *E. coli*.

These strains were all cultivated successfully by spreading the non-nutrient agar with live *E. coli*.

The *E. coli* used to prepare all plates was a 'wild' strain from the bacteriological sampling programme carried out at the same laboratory, and was maintained on plate count agar. To check against accidental contamination, it was partially characterized periodically, by testing its ability to ferment lactose at 37°C in the presence of bile salts (fermentative growth on MacConkey Agar).

Reference cultures of a few large amoebae, such as *Mayorella* and *Thecamoeba*, were maintained to aid identification of amoebae from field samples (see Section 3.5). These amoebae feed on smaller protozoa or algae, rather than bacteria, and require very moist culture conditions. They were grown as sealed plate cultures with *E. coli* and a smaller amoeba, usually *Hartmannella vermiformis*, as food.

Trophozoites from continuous axenic cultures (ie. grown in a nutrient medium without a food organism) were used for growth rate experiments (Section 2.8). The composition of Chandler Fulton's Medium A (CFA), modified by the addition of an antibiotic cover, is shown in Table 2.1 (after Fulton, 1970). CFA was also used to grow trophozoites for disinfection experiments, to avoid the heterogeneity of plate cultures, which might have cells in various stages of encystement. Clean plate cultures were used to initiate axenic cultures of some field isolates of *Naegleria* for pathogenicity or serological tests in laboratory mice, which were carried out by the Amoebic Research Unit, Institute of Medical and Veterinary Science, Adelaide or the Immunology Department, Adelaide Children's Hospital. The antibiotic cover was particularly useful to control any residual contamination by live bacteria from the water sample.

TABLE 2.1. FULTON'S MEDIUM A (CFA)

(after Fulton, 1970)

COMPONENT		CONCENTRATION	
		STOCK	FINAL (in CFA)
1	PHOSPHATE BUFFER		
	KH_2PO_4	3.4 g	10 mM with resp. to phosphate
	$NaHPO_4.7H_2O$	6.7 g	
	RO/DI water*	to 100 ml	
2	DEXTROSE SOLUTION		
	Dextrose	13.5 g	30 mM
	RO/DI water	50 ml	
3	HL-5 BASE		
	Proteose-peptone (Difco)	10.0 g	1.0%
	Yeast extract (Difco)	5.0 g	0.5%
	RO/DI water	650 ml	
	Distributed in 65 ml aliquots		

Components 1 to 3 were autoclaved separately. When they were cool, HL-5 was prepared by adding 2 ml phosphate buffer and 2 ml dextrose solution to each aliquot of base. HL-5 was stored at 2^{o} to $5^{o}C$, where it is stable indefinitely (Fulton, 1970).

FULTON's MEDIUM A		
HL-5	69 ml	
Foetal calf serum (CSL)	10 ml	
Penicillin/Streptomycin stock + DO/DI water	21 ml	500 u/ml pen. 500 µg/ml strep.

* water purified by Reverse Osmosis and Deionization

2.3 COLLECTION AND PROCESSING OF FIELD SAMPLES

Since many amoebae, including *Naegleria* and *Acanthamoeba*, produce cysts which are resistant to desiccation and may occur in airborne dust, water samples were collected in a manner which minimized outside contamination. Equipment and procedures for collecting water samples aseptically are well established through bacteriological studies.

All samples were collected in clean, sterile glass bottles (capacity 250 ml) with ground glass stoppers covered by an aluminium foil 'skirt' which reached the shoulder of the bottle. The skirt prevented contamination of the mouth of the bottle during handling. For samples of chlorinated water, a volume of sodium thiosulphate ($Na_2S_2O_3$) solution sufficient to neutralize approximately 12 mg/l chlorine (American Water Works Association, 1976) was added to the bottle before autoclaving.

Samples from water bodies such as the reservoirs and the River Murray were collected with the bottle held in a stainless steel sampling stick which could be flame sterilized immediately before use. Samples from points on the water supply after chlorination and from some reservoir outlets were collected from especially installed taps. The taps were regulated by a brass needle valve, avoiding the use of washers which might allow growth of microorganisms, and included a curved stainless steel nozzle approximately 120 mm long which could be flame sterilized. The whole tap was covered by a hinged box to minimize contamination by dust.

In collecting a sample, the tap was flushed for several minutes, flame sterilized using a portable gas torch, then run briefly before sampling. The design of the tap and the procedure described ensured that any microorganisms isolated were representative of the quality of water in the main at the time of sampling.

Field measurements of water temperature and free and total chlorine residual (where appropriate) were made as close in time as possible to collection of the microbiological samples. Samples were returned to the laboratory with a small air space and at ambient temperature, since low temperatures are lethal to trophozoites of *Naegleria fowleri* (Chang, 1978; see also Section 4.3).

At the laboratory, samples for isolation of amoebae were processed by a method adapted from Jamieson and Anderson (1973). A sample was filtered aseptically through a sterile cellulose nitrate membrane filter with mean pore size 8 μm and diameter 47 mm (Sartorius SM 11301). The membrane filter had been prewashed with 100 ml sterile distilled water to remove any detergent remaining from the manufacturing process. A fresh sterile filter cup (glass: Millipore Corp. or stainless steel: Sartorius) was used for each sample to prevent cross contamination.

The material deposited on the filter was resuspended in 0.7 ml quarter-strength Ringer solution, using a sterile wire loop. The whole volume was inoculated onto 5 agar plates spread with heat-killed *E. coli* (Section 2.2) and the membrane itself inverted onto a sixth. The plates were incubated as duplicates at 20, 28, and 37°C, with the plate including the membrane as one of the pair at 28°C.

Plates were examined under a stereomicroscope after 3 days (37°C), 4 days (28°C) and 5 days (20°C) incubation (Section 2.5), and presence or absence of amoebae at each incubation temperature recorded.

Late in the survey, after all *Naegleria* strains whose temperature tolerance had been determined grew at 30°C or above, incubation of primary cultures at 20°C was discontinued and incubation of plates at 42°C was included. The usefulness of the range of incubation temperatures will become clear in Section 4, which examines the role of temperature in the biology and distribution of *Naegleria*.

Each sample was assigned a unique number which was cross-referenced to a location number for those locations sampled regularly during the survey. Any amoeba identified was subsequently referred to by the sample number and incubation temperature at which it was isolated: for example, *Tetramitus ampliporus* 13749/28, the type strain of a new species described in Section 3.3.2.

2.4 MEASUREMENT OF CHLORINE RESIDUAL

Chlorination is the most widely accepted method of disinfection of water, and is used almost exclusively in Australia. The most important field parameter in managing chlorination of a public water supply or swimming pool is the chlorine residual, an instantaneous measure of chlorine concentration at any time after application of the chlorine dose. The chlorine residual is a dynamic parameter, varying with contact time and with physical and chemical characteristics of the water. Important aspects of the chemistry of chlorine in water are discussed in Section 5.2.

For the field study presented in Section 5.6, chlorine residual was measured at the time of collection of each sample. The method of choice in the field was titration against 0.00564 N phenylarsine oxide, with the end-point measured amperometrically (American Water Works Association, 1976). An especially designed and calibrated 'chlorine titrator' (Wallace and Tiernan or Fischer and Porter) was used.

Occasionally it was necessary to use the Lovibond Comparator, a test kit based on the red colour developed by dimethylphenylenediamine (DPD) in the presence of chlorine. Intensity of the colour was matched against a colour wheel, and the matching was somewhat subjective since the wheel colours do not represent a continuous range of concentrations.

The preferred field method, using the amperometric titrator, requires a sample volume of 200 ml. For laboratory-scale disinfection experiments (Sections 5.3-5.5), a modification of the ferrous/DPD method (American Water Works Association, 1976) was used to allow use of a small sample volume. A 20 ml sample was added to 10 ml 8.05 mM DPD solution buffered at pH 7.0. The red colour developed was titrated to a colourless end-point using 0.56 mM ferrous ammonium sulphate. At this concentration of the titrant, the titre end-point (in ml) was numerically equal to the chlorine residual (in $mg.l^{-1}$).

In each of the methods, the initial titration (or colour reading) provides a free chlorine residual. Measurement of chlorine present in a combined form (as chloramines) was made by adding excess potassium iodide (KI) and adjusting the pH to 4.0. Additional free chlorine displaced under these conditions was titrated to provide the total chlorine residual. The significance of the free and combined chlorine residuals is discussed in Section 5.2.

Using amperometric titration, concentrations as low as 0.1 $mg.l^{-1}$ could be measured, and field titrations were expressed in intervals of 0.1 $mg.l^{-1}$. Concentrations as low as 0.05 $mg.l^{-1}$ were measured by the ferrous/DPD method. In both methods, lower concentrations could be detected without accurate measurement.

2.5 MICROSCOPY AND PHOTOMICROGRAPHY

After completion of the incubation time appropriate to each temperature (Section 2.3), plate cultures prepared from field samples were examined at 50 times magnification using a Wild stereomicroscope with oblique sub-stage illumination. Presence or absence of amoebae was recorded for each plate, and amoebae were selected for detailed identification on the basis of plaque form and presence or absence and size of cysts.

All manipulations of amoebae for identification, subculture or cloning took place under the Wild stereomicroscope (Section 2.1).

The morphology of trophozoites and cysts of Vahlkampfiid amoebae, of *Acanthamoeba* species and of other amoebae of interest was examined in sterile Ringer solution using phase contrast microscopy at magnifications of 500 to 1260 times. A Carl Zeiss Standard or a Carl Zeiss Photomicroscope III was used for all high-power observations. Amoebae were identified using criteria discussed in detail in Section 3.

Photomicrographs (Figures 3.1 to 3.42) were taken at 800 to 1260 times magnification through a 63 X planapochromat lens, using the automatic exposure provided by the Carl Zeiss Photomicroscope III. All exposures were made on high speed black and white film (Ilford HP-4 or HP-5).

2.6 AMOEBA-FLAGELLATE TRANSFORMATION

The transformation of trophozoites to flagellates is a characteristic and controllable feature of most *Naegleria* strains. While the transformation has been studied as a model of cell differentiation (Fulton, 1970), it was used here as an important criterion distinguishing *Naegleria* species from other Vahlkampfiid amoebae (see Section 3.3.1).

The stimulus for transformation is a reduction in the osmolarity of the medium (Fulton, 1970). To test for the ability of a new Vahlkampfiid isolate to transform, trophozoites were suspended from the primary plate in a drop of sterile distilled water. The trophozoites were allowed to settle on a coverslip and the drop sealed to a brass ring on a microscope slide, using petroleum jelly. The morphology of the trophozoites was checked and the sealed drop incubated at 28°C or 37°C for up to 3 hours. The rate of transformation is temperature-dependent and some authors recommend testing at 37°C only.

However, in this study many *Naegleria* isolates were not tolerant of temperatures above 33° or 35°C (see Section 4.2). The incubation temperature chosen for the test was based on the temperature of isolation. For most *Naegleria* isolates, flagellates were present within one hour at 37°C or within two hours at 28°C.

Using a mouth-operated glass micropipette, single flagellates were drawn from the drop and used to initiate clonal cultures on heat-killed *E. coli*. This was the most practical method of ensuring a pure culture, since the primary plate often carried a mixed culture of amoebae; sometimes more than one Vahlkampfiid, or another highly motile amoeba such as *Acanthamoeba*.

A few other Vahlkampfiid amoebae are able to transform to flagellates (see Section 3.3.2). In the study described here they were collectively far less common than *Naegleria* species, were readily distinguished by the structure of their cysts, and did not transform in the times allowed in the test for *Naegleria*. For example, *Paratetramitus jugosus* (the most commonly encountered of these) required 24 to 36 hours for the appearance of flagellates in the few extended tests which were performed.

Longer incubation times were also used in tests carried out to determine the generic position of two previously undescribed Vahlkampfiid amoebae (see Section 3.3.2, 3.3.3).

2.7 MEASUREMENT OF TEMPERATURE TOLERANCE

The temperature tolerance of individual *Naegleria* and *Acanthamoeba* isolates was determined by incubating cultures simultaneously at a range of temperatures from 28°C to 44°C. Twenty or more trophozoites were used to initiate cultures on non-nutrient agar spread with heat-killed *E. coli* in 36 mm petri dishes. The criterion

for growth at a particular temperature was an increase of 10 mm in the radius of the plaque formed in the bacterial lawn. To help judge this growth, concentric arcs of 5 and 15 mm radius were scribed, centred on the edge of the plate. The inoculum was placed entirely within the inner arc, and the isolate was considered to have grown when the first trophozoites reached the outer arc.

Cultures were examined intially after 48 hours incubation and growing cultures which had still to fulfil the growth criterion were replaced. Trophozoites rarely survived a temperature 2°C above the highest temperature at which active growth occurred. Occasionally trophozoites survived without undergoing more than a few divisions, or were able to encyst. To test for survival, a drop of *E. coli* suspension was added to the plate which was reincubated at a lower temperature and the original growth criterion applied.

The growth criterion was not exactly equivalent for *Naegleria* and *Acanthamoeba*, since *Acanthamoeba* were more motile on agar and reached the outer arc after fewer divisions.

2.8 MEASUREMENT OF GROWTH RATES

In Section 4.3, the influence of temperature on growth rates of various strains of *Naegleria* is examined.

The initial density of amoebae growing axenically in Fulton's medium A (CFA) was adjusted to allow at least 24 hours of log-phase growth over the range of incubation temperatures to be used. Cultures were mixed carefully and the amoebae counted using a bright-line haemocytometer. Five or more replicate counts were made, usually totalling at least 200 organisms, and a mean and variance calculated. After 24 hours incubation, the amoebae were recounted by the same method, and the relative number of organisms, $\frac{n_{24}}{n_0}$, calculated.

Where it was impractical to count amoebae after exactly 24 hours incubation, particularly for the slower growth rates, the ratio $\frac{n_{24}}{n_0}$ was calculated from the following formula:
For an asynchronously dividing population of cells,

$$\log \frac{n_{24}}{n_0} = \frac{24}{t} \log \frac{n_t}{n_0}$$

The mean generation time, g, was calculated from the formula

$$\frac{t}{g} = \frac{\log \frac{n_t}{n_0}}{\log 2}$$

where t = incubation time; n_0 = initial numbers and

n_t = numbers at time t.

From the ratio $\frac{n_{24}}{n_0}$, the rate constant, k_{24}, and its standard deviation were calculated and plotted against temperature.

$$\text{In general, } k = \frac{\log \frac{n_t}{n_0}}{t.\log 2}$$

For numbers counted at (or corrected to) 24 hours and t expressed in days (=1),

$$k_{24} = \frac{\log \frac{n_{24}}{n_0}}{\log 2}$$

is numerically equal to the number of generations in 24 hours. The assumption of asynchronous division applies to all the preceding derivations.

In experiments described in Section 4.3, k_{24} increased linearly with temperature to within approximately $2^{\circ}C$ of the temperature optimum. Points from the fitted regression line were used to calculate the temperature coefficient,

$$Q_{10} = \left(\frac{k_2}{k_1}\right)^{\frac{10}{T_2 - T_1}}$$

where T_1, T_2, are two incubation temperatures.

$$\text{i.e. } Q_{10} = \frac{k_2}{k_1}$$

for T_1, T_2 which are $10^{\circ}C$ apart.

The temperature coefficient of growth in experiments with *Naegleria* species varied with the temperature interval chosen. In order to compare temperature coefficients for different strains, it was necessary to choose a standard temperature interval for calculation of Q_{10} values. The temperature range over which growth occurred varied greatly between *Naegleria* isolates from the field sampling, and between reference strains of *N. fowleri* and *N. gruberi* (Section 4.2). However, the range overlapped at least from 20° to $30^{\circ}C$ for all isolates studied, and temperature coefficients were calculated for this interval (see Table 4.3).

2.9 PLAQUE COUNT METHOD

A method was developed for counting viable trophozoites or cysts of *Naegleria* by the formation of plaques in a lawn of bacteria. The method was particularly useful in assessing survival in disinfection experiments (Section 5), where other authors have used a microscopic assessment of survival (Stringer and Krusé, 1971) or an 'extinction' method (Derreumaux *et al.*, 1974).

The suspension to be counted was thoroughly mixed and 1 ml added to a 2 ml overnight culture of *E. coli* in plate count broth. The *E. coli/Naegleria* suspension was mixed and 1 ml added to duplicate 2 ml aliquots of sloppy plate count agar.* The sloppy agar was mixed carefully, to avoid bubbles, and poured as an overlay onto level non-nutrient agar plates. These plates provided a duplicate count of viable *Naegleria* in the suspension.

* 0.7% agar

At the same time, a sample of the suspension was used for serial dilution. One ml was added to 2.16 ml of Ringer solution (previously dispensed) to make a 'half-log', i.e. 3.16-fold, dilution. Four serial half-log dilutions were prepared and duplicate plates poured from each. With the 'undiluted' plates, these provided a series of plaque counts at 10^{0}, $10^{-\frac{1}{2}}$, 10^{-1}, $10^{-3/2}$ and 10^{-2}. The 10^{0} count was actually a 3-fold dilution of the number of *Naegleria* in the original suspension, owing to dilution with the *E. coli* plate count broth. The actual numbers in the suspension were not usually calculated, since a relative count, such as 'percentage survival' was usually required. The plates with their overlays were incubated (at 37°C for most strains) and a cumulative count of plaques made as they appeared, usually after 2, 3 and 4 days incubation. The use of a sloppy agar overlay limited the motility of the amoebae, giving smaller, sharper plaques with little overlap error. The plaque count method used by Fulton (1970), without agar in the overlay, was only useful up to about 30 plaques per plate.

All volumes (samples, diluent, broth, etc.) were dispensed using an Oxford pipettor, the calibration of which had been checked (reproducibility ± 1 percent or better) before use. The unusual volumes for the half-log dilutions were dispensed using an Oxford Macroset, variable 1 to 5 ml; the volume was preset by the weight of distilled water dispensed.

The dilution interval was determined by the limits of acceptable variance and acceptable overlap error. For plaque count methods in general, a Poisson distribution ($s^2 = \bar{X}$) is predicted (Howes and de St. Groth, 1969), assuming random distribution of 'plaque-forming units' (i.e. adequate mixing) in the suspension to be assayed. Thus for a mean plaque count around 25, average standard deviation would be approximately 20% of the mean and would increase at

lower counts. Although overlap errors were not formally calculated, mean plaque counts in successive dilutions generally gave good agreement up to approximately 200 plaques per plate, provided that the first count was made early, when plaque diameters were about 1 mm. For estimating the number of viable *Naegleria*, the dilution with a final count between 30 and 200 plaques and with the best replication was usually accepted. The half-log dilution interval ensured that there was at least one count in this range, except at very low percentage survival.

Acanthamoeba species were rather more motile in agar than *Naegleria* and produced irregular plaques with 'satellites' when a sloppy overlay was used. For the disinfection experiments on *Acanthamoeba*, an overlay of full-strength (1.4%) plate count agar was used to reduce overlap error, and a plaque count between 30 and 100 chosen wherever possible.

3. IDENTIFICATION OF AMOEBAE

3.1 INTRODUCTION

In this section, considerable care will be taken to establish the accuracy of identifications of amoebae from field samples. In later sections, laboratory studies of the effect of temperature on amoebae (see 4.2) and on control of amoebae by disinfection (see 5.3, 5.4) are presented. Well-characterised reference strains of *Naegleria* and *Acanthamoeba* were used in most of the experiments and comparison is made with the results of field sampling. Clearly, the validity of the comparison depends on accurate identification of the field isolates.

Two books dealing with the taxonomy and biology of amoebae have been published in recent years. Singh (1975) and Page (1976) differ in their approach to classifying amoebae; in particular, they disagree on the validity of names in *Naegleria* and *Acanthamoeba*, the genera of free-living amoebae which may be medically important. Thus a discussion of the taxonomy of amoebae is necessary to justify the nomenclature which is used and to explain the criteria used in identifying amoebae.

3.2 TAXONOMY OF AMOEBAE

There have been two general approaches to the taxonomy of amoebae which have been referred to as the 'pseudopodial approach' and the 'cytological approach' (Page, 1967a). The older descriptions employed the morphology and movement of amoebae, often emphasizing characters which were not a permanent feature of the cell. Poor descriptions and illustrations and inaccurate measurements led to extensive synonomy, and many of the names erected cannot be identified with any of the amoebae seen by recent taxonomists (Page, 1967a). Studies of amoebae in recent years have benefited from the introduction

of concepts and techniques from microbiology. Examples are the use of genetically uniform cultures or clones, availability of reference cultures including type cultures from culture collections, and cultivation using reproducible media and known food organisms.

In spite of the efforts of Schaeffer (1926) and Bovee (1965) to base descriptions and a classification on detailed observations of live, active amoebae, the 'pseudopodial approach' fell into disrepute. Singh (1975, ch. 3), in particular, believes that '...pseudopodial characters and movement in amoebae ... are insufficiently stable for diagnostic purposes'.

Using pure cultures of amoebae isolated from soil, Singh (1952) provided descriptions and a classification based largely on the cytochemical structure of the nucleus during mitosis (the 'cytological approach'). The promitotic division characteristic of the Vahlkampfiid genera certainly warrants the familial status given the group by both Singh (1952) and Page (1967a). However, the nuclei of other free-living amoebae vary less, and share '...a common mitotic pattern resembling that found throughout the animal kingdom' (Page, 1967b).

Although Singh distinguishes some species by the structure of the cyst, his classification discusses only one genus outside the Vahlkampfiidae. The extreme of the 'cytological approach' is Kudo's contention that the number of nuclei is the only valid generic character in the free-living amoebae, and that '...the genus *Amoeba* should be retained and all free-living, typically uni-nucleate amoebae should be placed in it' (Kudo, 1959).

The classification developed over several years by Page, culminating in his 'Illustrated Key' (Page, 1976), is a synthesis of various approaches including the observation of live, 'locomotive' amoebae under natural conditions. Page has carried out detailed cytochemical studies (e.g. Page, 1967a, b) and these characters are

considered in his placement of species and genera. However, in most cases they are not required for identification as they are in Singh's classification.

A major reason for preferring the Page classification to that of Singh is that it is practical. Amoebae belonging to 21 genera recognized by Page were identified during this study (see Table 3.2) without making large numbers of stained preparations. Singh's (1975) classification encompassed a relatively small number of species; nevertheless few of them, apart from the *Acanthamoeba* species and some of the Vahlkampfiids, could be identified with any of the amoebae seen in this study. The narrow base of cytological characters makes Singh's descriptions essentially impractical.

A second serious reason for rejecting Singh's classification is that his nomenclature in the genera *Naegleria, Vahlkampfia* and *Acanthamoeba* does not follow the Rules of Zoological Nomenclature (see Sections 3.3 and 3.4).

TABLE 3.1

DESCRIBED GYMNAMOEBIA ('NAKED AMOEBAE') IDENTIFIED IN THE SAMPLING STUDY. CLASSIFICATION AFTER PAGE (1976).

FAMILY	SPECIES
AMOEBIDAE	*Polychaos* species
VAHLKAMPFIIDAE	*Vahlkampfia aberdonica*
	V. atopa
	V. avara
	V. inornata
	V. russelli
	V. ustiana
	Naegleria species (see Section 3.3.1)
	Adelphamoeba galeacystis
	Paratetramitus jugosus
HARTMANNELLIDAE	*Hartmannella vermiformis*
	Saccamoeba nr. *limax*
	Cashia angelica
	C. limacoides
COCHLIOPODIIDAE	*Cochliopodium actinophorum*
	Gocevia species
FLABELLULIDAE	*Rosculus ithacus*
THECAMOEBIDAE	*Thecamoeba granifera*
	T. striata
	T. terricola
	Vannella mira
	V. platypodia
	V. simplex
	Platyamoeba placida
	P. stenopodia
HYALODISCIDAE	*Flamella* species
ECHINAMOEBIDAE	*Echinamoeba exundans*
	Filamoeba nolandi
ACANTHAMOEBIDAE	*Acanthamoeba* species (see Section 3.4)
PARAMOEBIDAE	*Mayorella* species
	Vexillifera bacillipedes

3.3 THE VAHLKAMPFIIDAE

The family Vahlkampfiidae contains amoebae which move by eruptive, rounded, hyaline pseudopodia (Figures 3.1, 3.9 etc.) and which divide by promitosis. Promitosis, or 'intranuclear mitosis' (Fulton, 1977), is characterised by retention of the nuclear membrane throughout division and is unique to the Vahlkampfiidae. In addition, the nucleolus divides into two 'polar caps' and is recognizable throughout mitosis. Thus the family Vahlkampfiidae Jollos, 1917 corresponds exactly with the family Schizopyrenidae of Singh, 1952. Singh's refusal to recognize the name Vahlkampfiidae stems from his opinion on the validity of the nominate genus (see 3.3.3).

Five genera are characterized by the ability of the trophozoites to undergo transformation to flagellates. Of these, *Heteramoeba* has been described only from brackish water and will not be considered further. *Tetramitus* species have been described from sea- and fresh water and from faecal material (Page, 1976). *Tetramitus* species appear to have a relatively stable flagellate phase. Indeed, the type species was originally described as a flagellate, and an amoeboid stage has not been seen for some of the described species. Identification of *Tetramitus*, *Paratetramitus* and *Adelphamoeba* will be discussed in Section 3.3.2.

Naegleria species are the most commonly isolated Vahlkampfiid amoebae from fresh water. Page (1976) believes that *N. gruberi* is '... the most common freshwater amoeba and apparently world-wide in distribution'.

The genus *Vahlkampfia* contains all amoebae typical of the family which are unable to transform to flagellates.

Paratetramitus jugosus was originally described as a *Vahlkampfia* species (Page, 1967a), and was made type species of a new genus when the existence of a flagellate stage was discovered (Darbyshire *et al.*, 1976).

3.3.1 *Naegleria*

It is generally agreed that *Naegleria gruberi* is the type (and most common) species of a genus of Vahlkampfiid amoebae which transform more or less readily to flagellates which lack a cytostome and which usually have two flagella (Figures 3.2, 3.3). Genera erected solely on the basis of a different number of flagella (e.g. *Trimastigamoeba*) are synonyms of *Naegleria*. Page's (1967a) careful counting and measurement of hundreds of individuals of several strains demonstrated that flagellar number differed in up to 4% of flagellates; Fulton (1970) claimed that three or four flagella were even more common in certain strains; Dingle (1970) studied experimental conditions under which the number of flagella may vary.

Singh (1952) made the presence of interzonal bodies during mitosis an essential character of *Naegleria*. He established the genus *Didascalus* for Vahlkampfiid amoebae which divided without producing interzonal bodies and which transformed to flagellates 'less readily' than the strains of *Naegleria* studied.

However Page (1967a) claimed that interzonal bodies are not invariably present in division figures even of one strain of *N. gruberi*, and Carter (1970) was unable to identify them in *N. fowleri*. Singh (1975) later claimed that interzonal bodies were present in one of Carter's figures. Clearly, the presence of interzonal bodies is a matter of cytochemical technique and interpretation of stained mitotic figures. It should not be required for accurate identification.

Didascalus is considered here to be a synonym of *Naegleria*. Slow or low percentage transformation alone is not sufficient to establish a separate genus or even species. Fulton (1970) described the selection of stable mutants of *N. gruberi* which transformed slowly and at a low percentage and of others which failed to transform altogether. It is reasonable to believe that such strains may occur

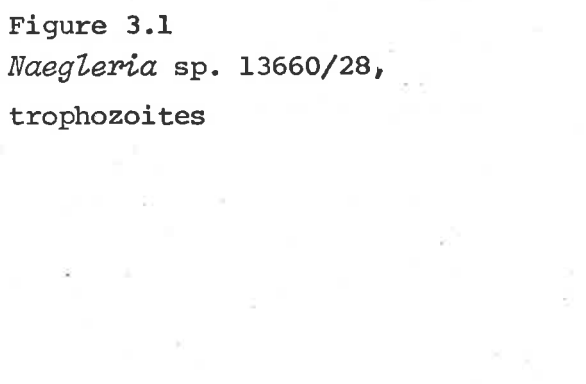


Figure 3.1
Naegleria sp. 13660/28,
trophozoites

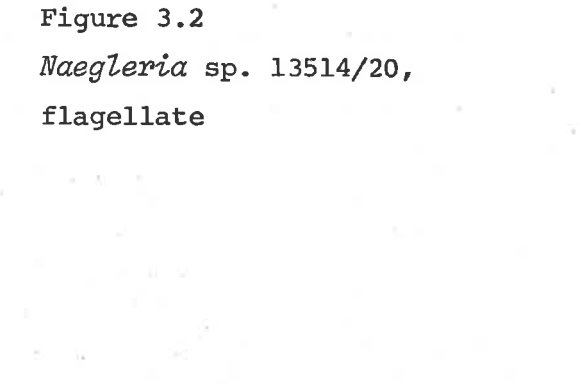


Figure 3.2
Naegleria sp. 13514/20,
flagellate

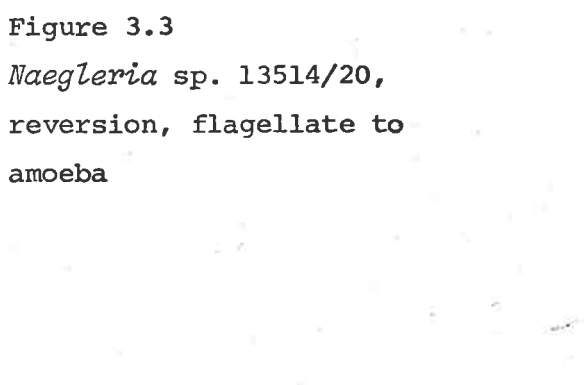


Figure 3.3
Naegleria sp. 13514/20,
reversion, flagellate to
amoeba

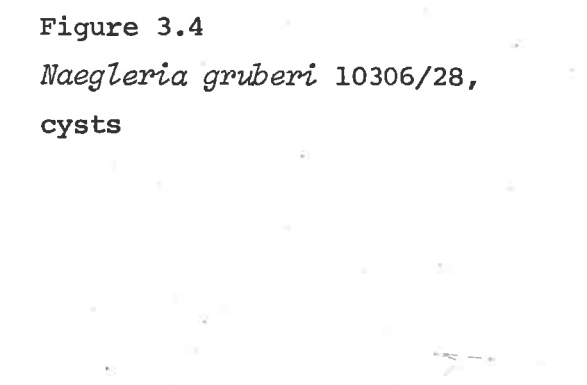


Figure 3.4
Naegleria gruberi 10306/28,
cysts

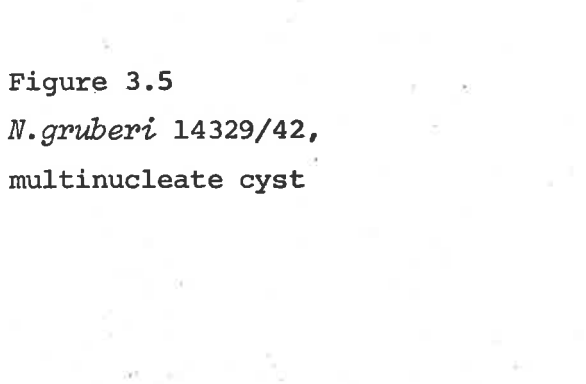


Figure 3.5
N.gruberi 14329/42,
multinucleate cyst

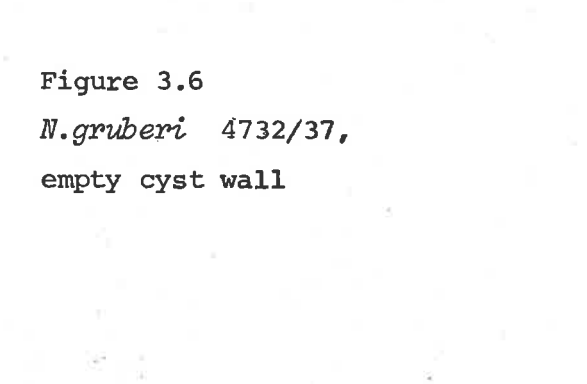


Figure 3.6
N.gruberi 4732/37,
empty cyst wall

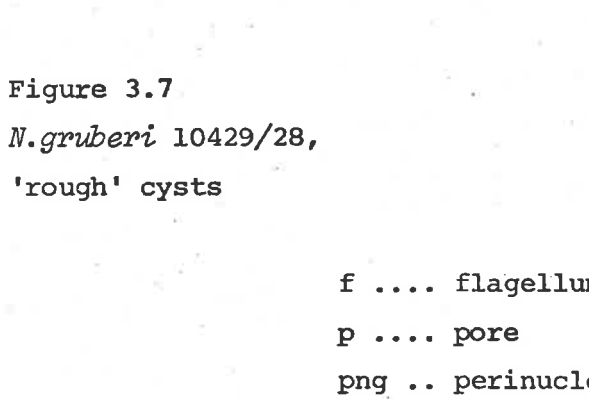


Figure 3.7
N.gruberi 10429/28,
'rough' cysts

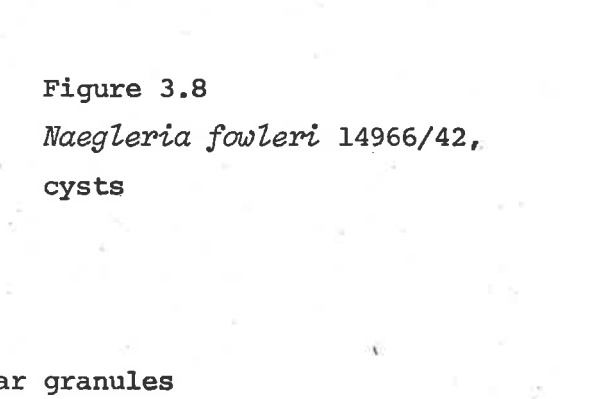
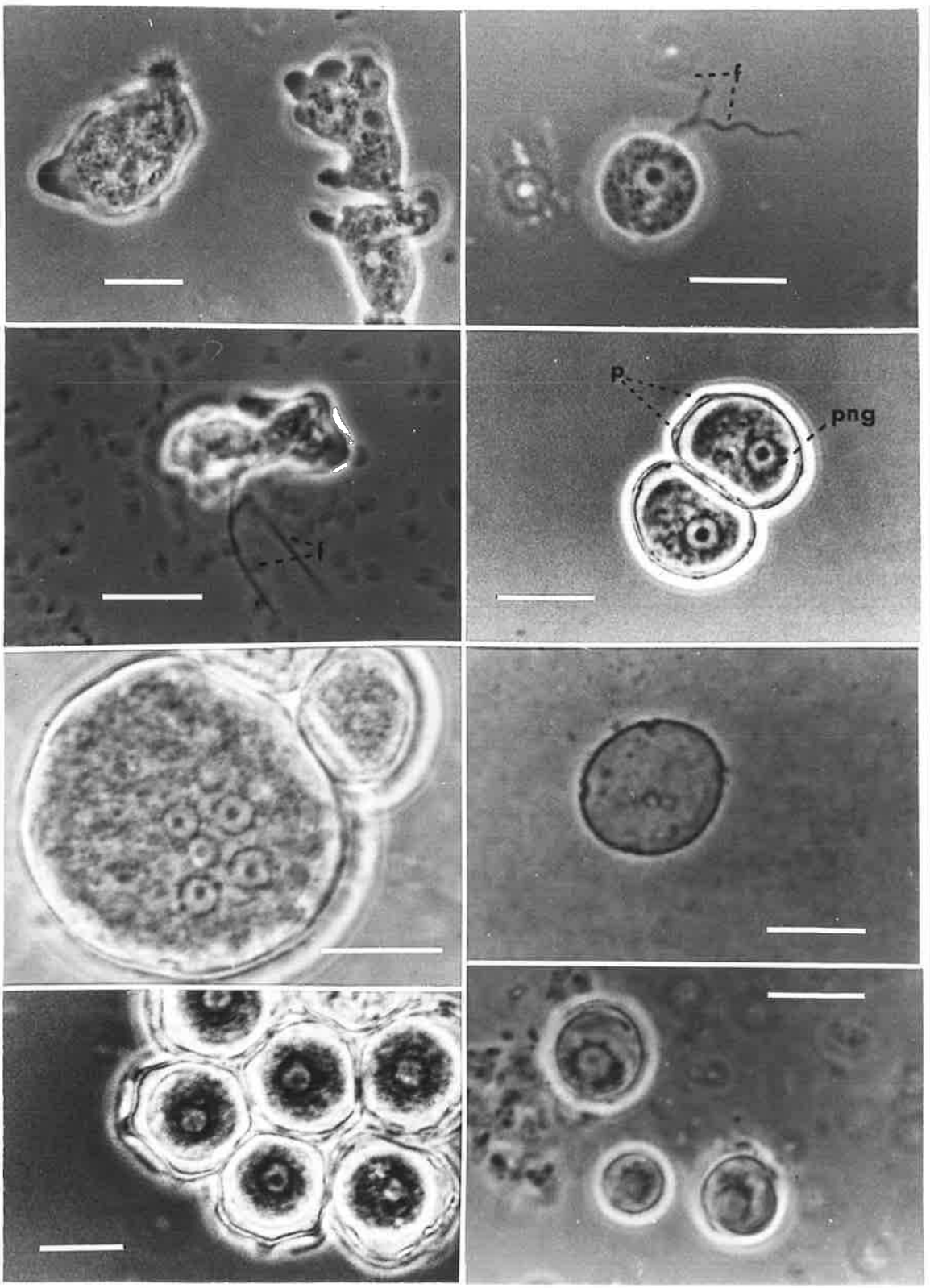


Figure 3.8
Naegleria fowleri 14966/42,
cysts

f .... flagellum
p .... pore
png .. perinuclear granules
Scale = 10 µm


f
f
p
png

naturally, varying little in other genetic respects from 'normal' *Naegleria* strains.

The readiness with which trophozoites underwent transformation varied greatly among the large number of *Naegleria* isolates identified in this study. Strains were occasionally encountered in which no more than one percent of trophozoites transformed even on repeated testing of a clone grown from a flagellate. These isolates possessed other characters of *Naegleria* described in detail by Page (1967a), such as pores in the wall of the cyst and perinuclear granules. Such isolates were not always identified as *Naegleria* at the first test for transformation but a second test was performed after microscopical examination of the cysts (see 2.6).

Until the discovery that *Naegleria* could be pathogenic, the most detailed modern studies considered the genus monotypic (e.g. Singh, 1952; Page, 1967a). Carter (1970) described pathogenic strains from several human cases of primary amoebic meningoencephalitis and gave them specific status as *Naegleria fowleri*. Pathogenicity was the diagnostic character although other differences from available reference strains of *Naegleria gruberi*, including structure and size of the cysts, were noted.

A description of pathogenic *Naegleria* from a case in the United States of America, with the name *Naegleria aerobia*, appeared soon afterward (Singh and Das, 1970). Singh (1975), while admitting that the *Naegleria* species in the two descriptions were the same organism, claimed that Carter's description (specifically, his failure to observe interzonal bodies) placed it in the genus *Didascalus*. Under the rules of nomenclature, however, incompleteness of a description does not overrule priority of a name, particularly if the type material (in this case, type culture) is still available for study.

Pathogenicity correlates with other characters in the isolates which have been studied in detail. They are antigenically similar to each other and different from *N. gruberi* with very little cross-reaction (Willaert *et al.*, 1974). They differ from *N. gruberi* in the structure of the cysts produced, and are capable of growth at temperatures several degrees higher than 'typical' strains of *N. gruberi*. These generalizations are based on comparison with few strains of *N. gruberi*, but the characters of the pathogenic strains are uniform enough for the synonomy of *N. aerobia* with *N. fowleri* to be apparent.

Specific antisera against *Naegleria fowleri* were first prepared by Anderson and Jamieson (1972b) who used a slide agglutination test to study *Naegleria* species isolated 'from the environment'. Other studies have employed immunodiffusion (Visvesvara and Healy, 1975), immuno-electrophoresis (Willaert *et al.*, 1972), and fluorescent antibody technique (de Jonckheere *et al.*, 1974). These studies confirmed the distinctness of pathogenic *Naegleria* strains from reference strains of *N. gruberi*. However, in most of them, *Naegleria* strains were isolated which were not pathogenic to laboratory animals, but which could not be distinguished antigenically from *N. fowleri* using most techniques.

The taxonomic position of these isolates presented a difficulty. Some authors began to use the specific name *N. fowleri* for any antigenically similar strain, regardless of pathogenicity (de Jonckheere *et al.*, 1975). However, no formal emendation of Carter's criteria for *N. fowleri* was proposed. Further study of several isolates disclosed other common differences from *N. fowleri*, including electrophoretic pattern of several isoenzymes (Nerad and Daggett, 1979), the number of lines of precipitation on immunoelectrophoresis and the distribution of endoplasmic reticulum in the cyst (Stevens *et al.*, 1980). The latter authors have given this group of isolates specific

status as *Naegleria lovaniensis* (see further discussion in Section 4.2).

Cysts of *Naegleria gruberi* are usually rather regular in shape, circular to ovoid, with the ectocyst closely opposed to the endocyst (Figure 3.4). They are usually mononucleate with a prominent ring of perinuclear granules, but multinucleate cysts are common in some strains (Figure 3.5). *N. gruberi* cysts have pores which are readily visible by light microscopy. Scanning electron micrographs of one strain have shown each pore surrounded by a raised cone (Jadin *et al.*, 1974). The pores can be counted most easily in 'empty' cysts (Figure 3.6). In occasional 'rough' strains of *N. gruberi* the separation between endo- and ectocyst is irregular and the thickly plugged pores are even more prominent (Figure 3.7).

Carter (1970) and Singh and Das (1970) described the cysts of *N. fowleri* and *'N. aerobia'* as lacking pores (Figure 3.8). However, the presence of pores has since been demonstrated by transmission and scanning electron microscopy (Schuster, 1975; Jadin *et al.*, 1974). They are flat, less numerous than those in *N. gruberi*, and by light microscopy are easily seen only in empty cysts (de Jonckheere and van de Voorde, 1977).

A fourth group of *Naegleria* isolates, nonpathogenic and antigenically distinct from both *N. gruberi* and *N. fowleri*, has been given specific status as *Naegleria jadini* (Willaert and Le Ray, 1973). The structure of the cysts has been studied only in the type strain, and is similar to *N. fowleri* (Jadin *et al.*, 1974).

Griffin (1972) showed that nine isolates of *Naegleria fowleri*, all from human infections, could grow at 45^{o}C, while three reference strains of *N. gruberi* did not grow above 37^{o}C. Nearly 120 pathogenic isolates have not been studied in enough detail to confirm the uniformity of temperature tolerance inferred in Griffin's study. The few isolates

of *N. lovaniensis* which have been studied have similar temperature tolerances to that of *N. fowleri* (Stevens *et al.*, 1980).

The importance of temperature tolerance as a taxonomic tool and the influence of temperature on the biology of *Naegleria* species is discussed in greater detail in Section 4.

3.3.2 *Tetramitus*, *Paratetramitus* and *Adelphamoeba*

The flagellates of *Tetramitus* and *Adelphamoeba* have a cytostome, while those of *Paratetramitus* have a 'deep ventral groove' which may terminate in a cytostome, although this has not been demonstrated (Darbyshire *et al.*, 1976). These three genera (and *Heteramoeba* from brackish water) are differentiated by the position and structure of the cytostome, the structure of the cysts and the number of flagella: basically four in *Tetramitus*, two in *Paratetramitus*, *Adelphamoeba* and *Heteramoeba*. Although the presumed ability of the flagellates to feed suggests a less transient stage than the flagellate of *Naegleria*, the transformation is far less readily demonstrated in these genera.

During this study, isolates representing all Vahlkampfiid species identified were tested for transformation to flagellates (Section 2.6). However, the conditions were designed for recognition of *Naegleria* species, and extended tests were not usually performed. *Paratetramitus* (Figure 3.9, 3.10) and *Adelphamoeba* (Figure 3.11) were usually identified by the structure of their cysts. The cysts of *Tetramitus rostratus* (not identified in this study) are less distinctive, and this amoeba, if isolated, may have been mistaken for *Vahlkampfia inornata* (see Section 3.3.3) when flagellates were not observed.

Three isolates of a previously undescribed Vahlkampfiid which can transform to flagellates, although more slowly than most

Naegleria isolates, were made during this study. The new species is described here because it produces cysts with pores in the wall, and could easily be mistaken for *Naegleria* (which until now contained all valid Vahlkampfiid species which have cysts with pores). Furthermore, the three isolates studied grew rapidly at 44°C. This suggests that, like the recently described *Naegleria lovaniensis*, this species is likely to occur under conditions that also favour growth of *N. fowleri*.

Information concerning the samples from which the new amoeba was isolated is presented in Table 3.2. Dimensions cited in the diagnosis were based on measurements of 50 individuals from plate cultures grown with live *E. coli*. Transformation tests and measurements of amoebae were made using young (48 hour) cultures. Amoebae from cultures of the same age were stained by the Feulgen technique for demonstration of intranuclear mitosis. Measurements of cysts were made from older cultures.

The new Vahlkampfiid is assigned to *Tetramitus* because the flagellates have a prominent cytostome and (usually) four flagella. Its generic position is discussed in more detail following the diagnosis.

Tetramitus ampliporus n.sp. Figures 3.12-3.21

type strain : SWL 13749/28

type location : River Murray at Morgan, Sth. Australia, 29.8.79

other isolates : SWL 13613/42
SHLS 1900/43
(see Table 3.2)

Diagnosis

Amoebae in locomotion 22 to 45 µm (mean 35 µm) long, mean length/breadth ratio 1.8. Locomotion by eruptive hemispherical bulges from anterior ectoplasm. Usually a single discharging contractile vacuole, formed by coalescence of several smaller vacuoles. Prominent uroid present,

TABLE 3.2. SAMPLING DATA FOR REFERENCE STRAINS OF NEW VAHLKAMPFIID AMOEBAE

Reference No.	Location	Date	Water Temp. (°C)
Tetramitus ampliporus, n.sp.			
SWL 13749/28 (type)	River Murray at Morgan, South Australia	29.8.79	11
SWL 13613/42	Nelshaby Reservoir, South Australia	7.8.79	13
SHLS 1900/43	backwash water from swimming pool filter, Northam, W. Aust.	5.2.80	-
Vahlkampfia angularis, n.sp.			
SWL 8994/20 (type)	Happy Valley Reservoir, South Australia	4.7.77	10

SWL - State Water Laboratories, South Australia

SHLS - State Health Laboratory Service, Western Australia

Figure 3.9
Paratetramitus jugosus
13705/37 trophozoites

Figure 3.10
P.jugosus 13705/37,
cysts

Figure 3.11
Adelphamoeba galeacystis
9835/37, cysts

Figure 3.12
Tetramitus ampliporus n. sp.
13749/28, binucleate trophozoite

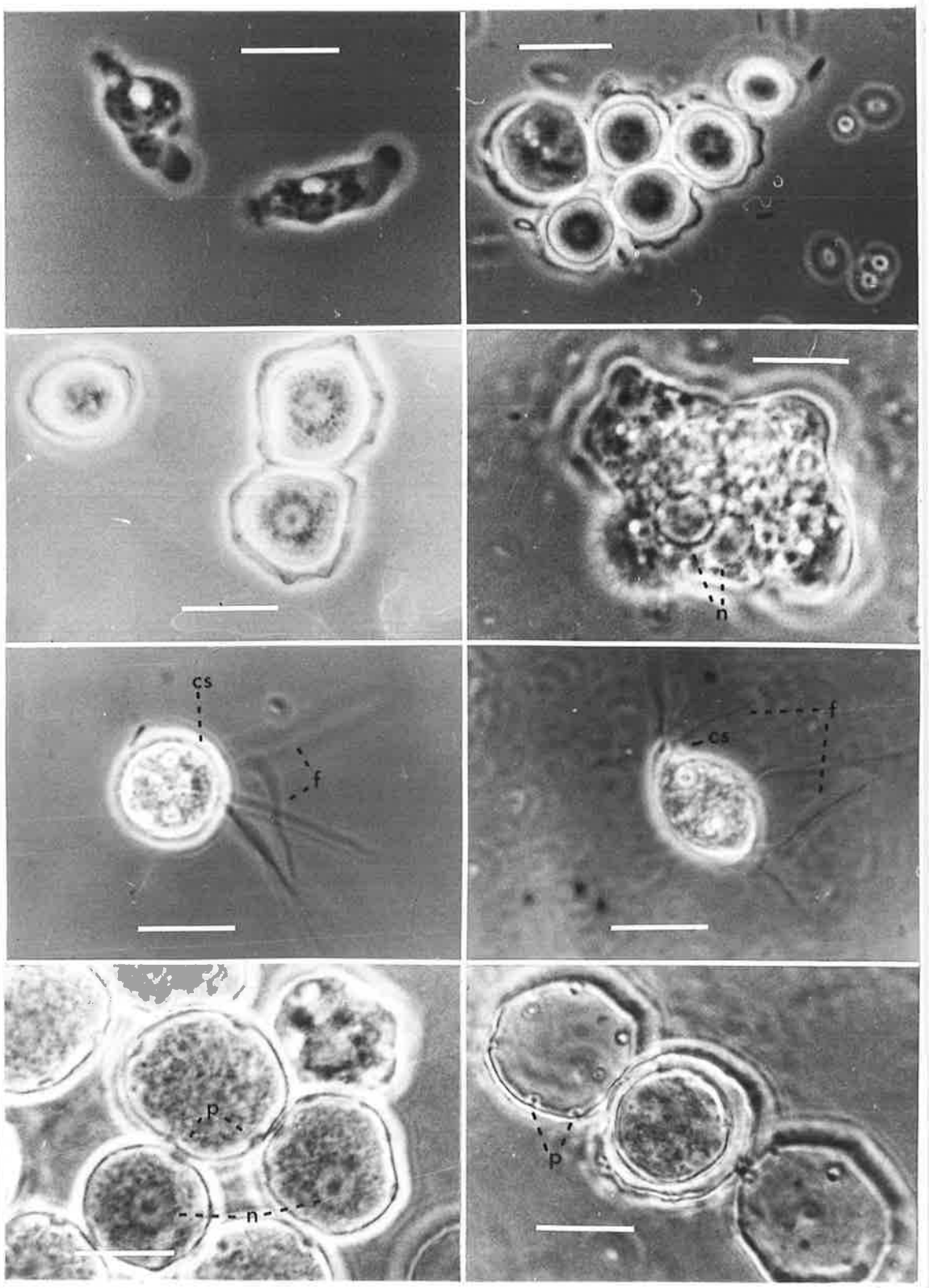
Figure 3.13
T.ampliporus 13749/28,
flagellate (probably dividing),
showing cytostome, 2 nuclei
and 2 groups of flagella

Figure 3.14
T.ampliporus 13749/28,
binucleate, bipolar flagellate

Figure 3.15
T.ampliporus 13613/42,
mature cysts

Figure 3.16
T.ampliporus 13749/28,
re-encystment

cs .... cytostome
f .... flagellum
n .... nucleus
p .... pore
Scale = 10 µm


n
cs
f
cs
f
p
n
p

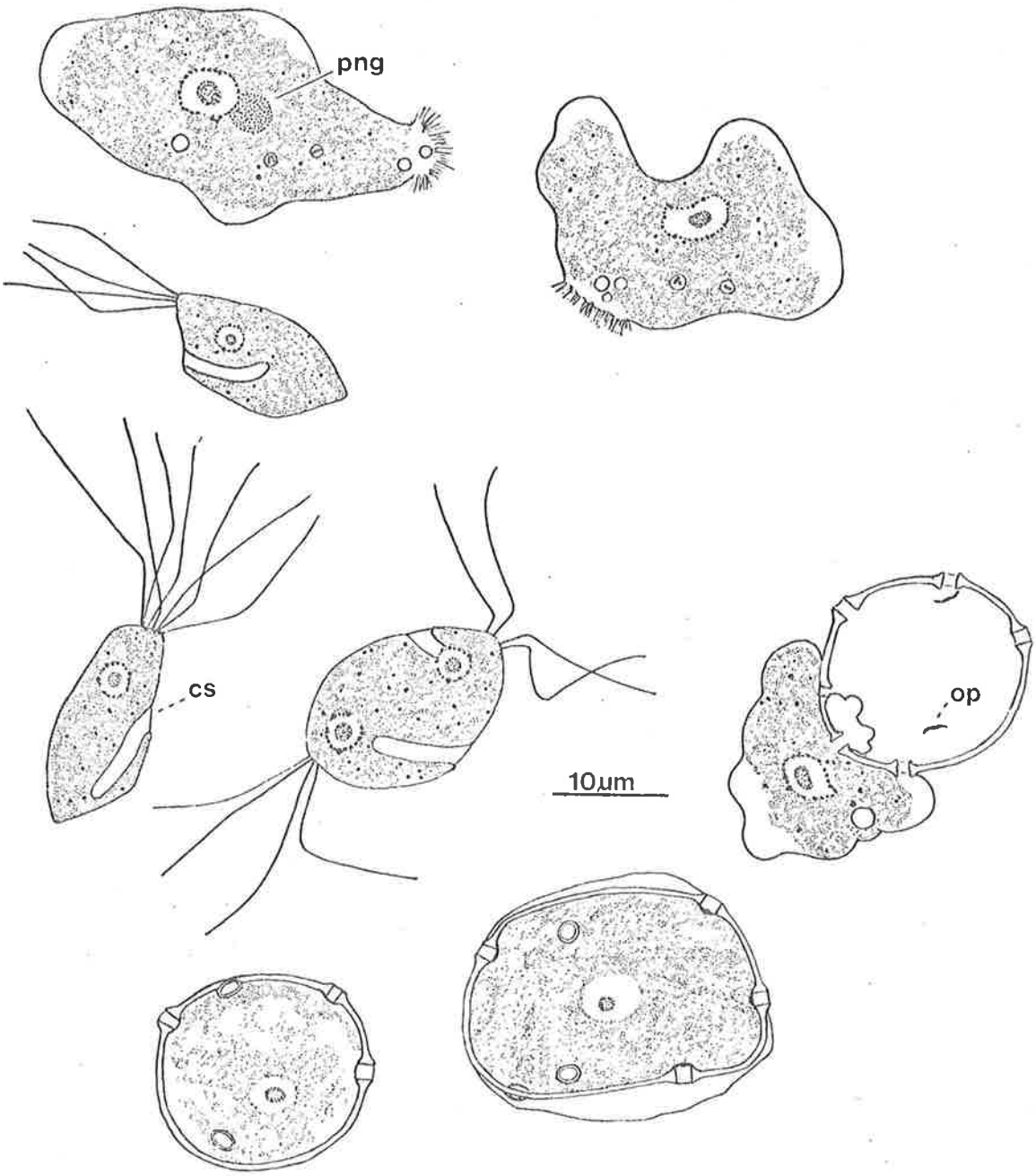


Figure 3.17 *Tetramitus ampliporus* n. sp.

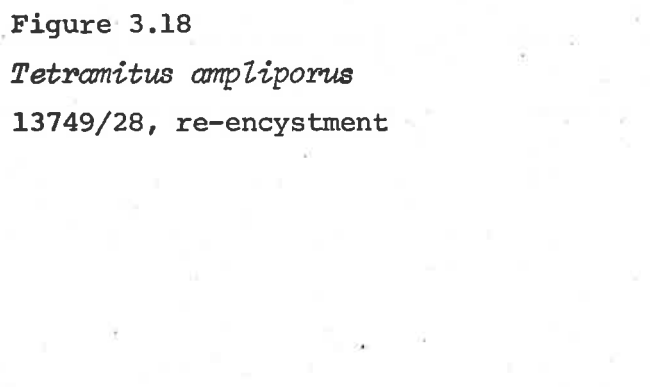


Figure 3.18
Tetramitus ampliporus
13749/28, re-encystment

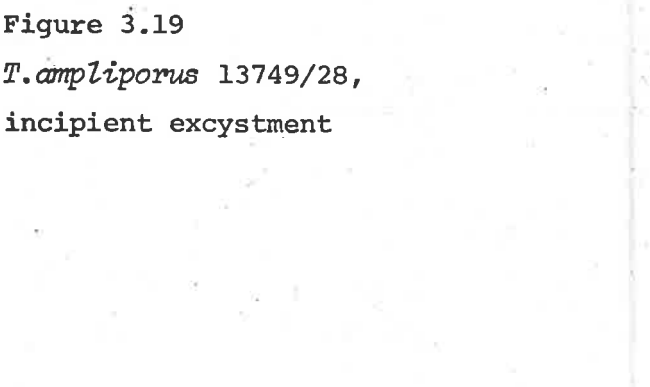


Figure 3.19
T.ampliporus 13749/28,
incipient excystment

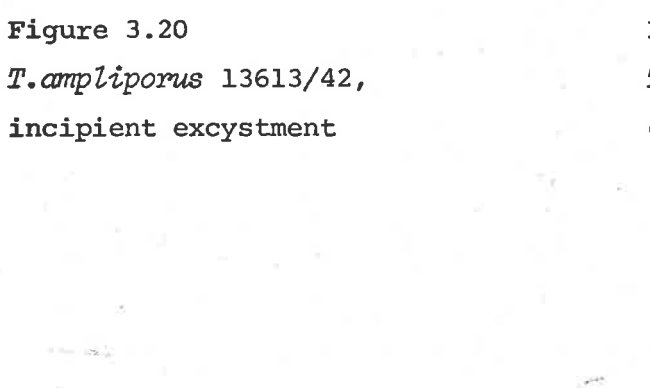


Figure 3.20
T.ampliporus 13613/42,
incipient excystment

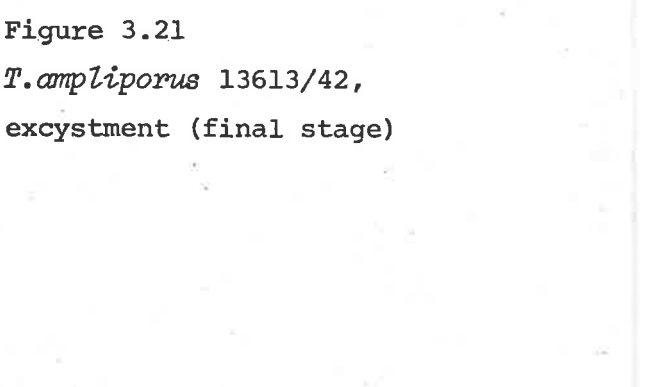


Figure 3.21
T.ampliporus 13613/42,
excystment (final stage)

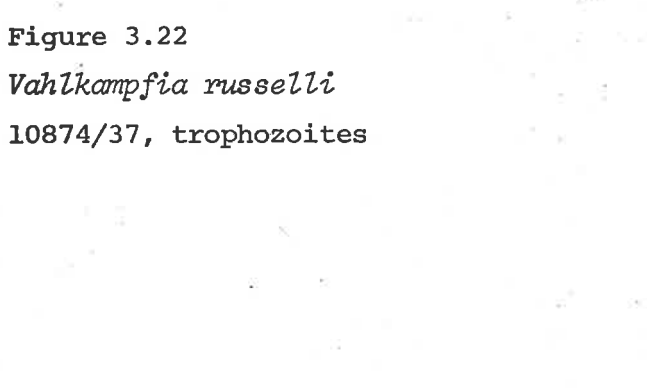


Figure 3.22
Vahlkampfia russelli
10874/37, trophozoites

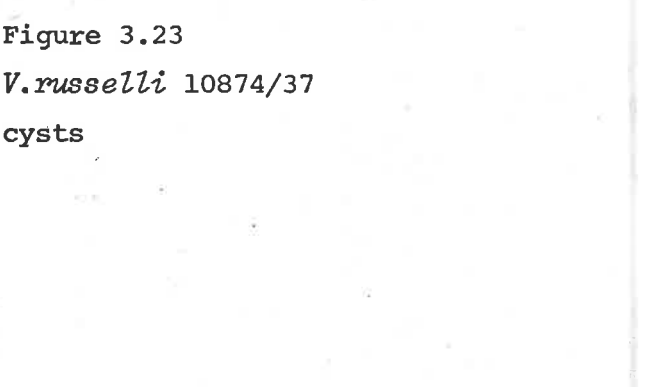


Figure 3.23
V.russelli 10874/37
cysts

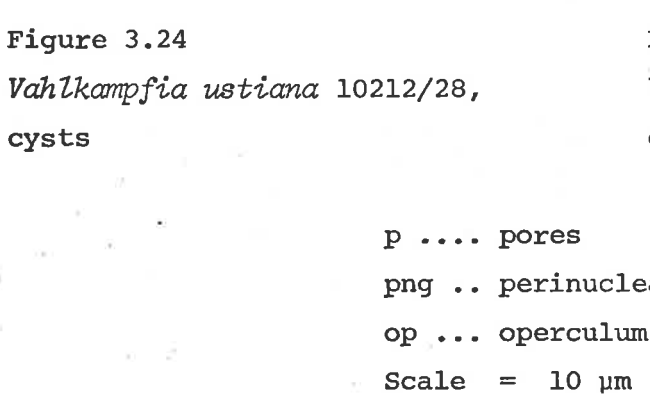


Figure 3.24
Vahlkampfia ustiana 10212/28,
cysts

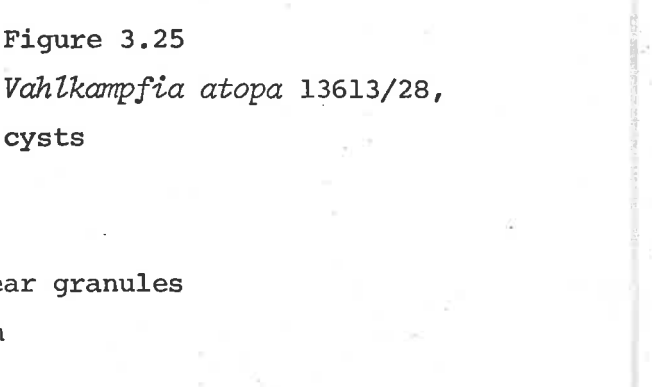
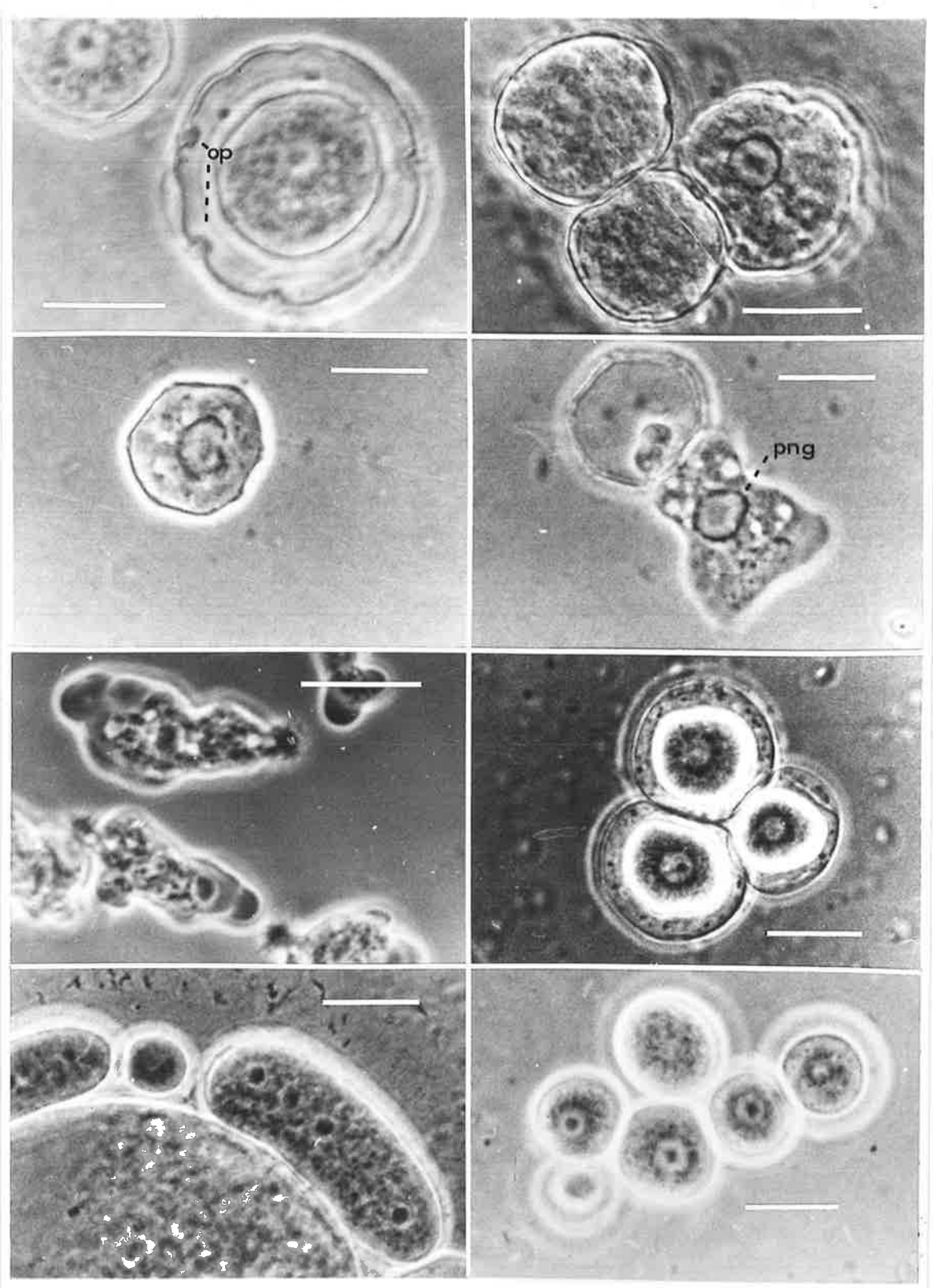


Figure 3.25
Vahlkampfia atopa 13613/28,
cysts

p .... pores
png .. perinuclear granules
op ... operculum
Scale = 10 µm


op
png

taking the form of a broad filamentous posterior margin or a villous bulb. Amoebae usually mono- or binucleate, but 3 or more nuclei common. Nucleus 5.1 to 8.9 µm (mean 7.2 µm) diameter, surrounded by a ring of refractile granules; large central nucleolus. Division by intranuclear mitosis.

Transformation to flagellates occurs in some strains. Flagellates approximately 15 to 22 µm long, usually mononucleate, but 2 or more nuclei common. Usually 4 to 8 equal flagella, slightly longer than the cell, inserted close together anteriorly in mononucleate flagellates. Cytostome long, curved, opening laterally. In binucleate flagellates, flagella inserted in groups of 4 to 8, often at opposite poles of the cell, occasionally with 2 cytostomes opening to opposite sides of the cell. Occasional flagellates with a higher number of nuclei may have flagella inserted at 3 or 4 points on the cell.

Cyst regularly or irregularly circular to ovoid, maximum dimension 12.2 to 28.0 µm (mean 17.8 µm), usually mononucleate. Ectocyst thin, smooth; loosely applied to ectocyst except at pores in some cysts, difficult to distinguish in others. Endocyst wall thick (approximately 0.6 µm), refractile. Excystment via one of numerous pores, usually 6 to 15, depending on cyst diameter; occasionally as few as 3 (mean 9.6). Endocyst thickened inward and raised into a prominent collar at each pore; diameter (including collar) approximately 2 µm. Refractile perinuclear granules absent in the mature cyst, but appear after excystment has been initiated. One or more saucer-shaped opercula usually visible inside vacant cyst wall following excystment.

Discussion

Identity of the three isolates:

The type strain transformed to flagellates under the same conditions used for *Naegleria* species (Section 2.6), although more slowly than most isolates of that genus. The first flagellates appeared after approximately 6 to 8 hours at 28°C. Isolates SWL 13613/42 and SHLS 1900/43, however, have not transformed to flagellates despite repeated tests with the type strain as a positive control.

Pending further study such as isoenzyme analysis, they are judged to belong to the same species by a number of common structural and physiological characters. All the features of the amoebae (number of nuclei, perinuclear granules, uroid process) and of the cysts (pore structure, loss and reappearance of perinuclear granules, persistence of operculum following excystment) in the diagnosis are common to the three isolates.

Re-encystment within the original cyst wall occurs, apparently in response to desiccation, in undisturbed cultures of each strain (Figure 3.18). Elsewhere in the Vahlkampfiidae, re-encystment has only been described in *Naegleria gruberi* where it occured in response to mechanical damage to the cyst wall (Chiovetti, 1976). The three strains have a uniform, high temperature tolerance (growth at 44°C).

Failure of some strains to transform to flagellates should not preclude their inclusion in a single species, since ability to transform is variable in *Paratetramitus jugosus* (Darbyshire *et al.*, 1976).

Generic position:

Assignment of *T. ampliporus* to *Tetramitus* is based on the number of flagella and the structure of the cytostome. The presence

of four to eight flagella at one site is interpreted as evidence that division may take place in the flagellate stage, or at least that nuclear division is not halted by transformation. Darbyshire *et al.* (1976) drew similar conclusions concerning *Paratetramitus jugosus*. Observation of a binucleate flagellate with the nuclei placed symmetrically about the axis of the cell and six flagella in two close groups of three, also symmetrically placed (Figures 3.13) supports this conclusion. Flagellates with more than one cytostome and flagella in two or more well-separated groups are assumed to have arisen from binucleate or multinucleate amoebae. In cultures of the type strain, amoebae with up to ten nuclei have been observed. More detailed studies are needed to confirm these assumptions.

The structure of the cyst of *T. ampliporus* suggests that this amoeba may be more closely related to *Naegleria* than any of the amoeboflagellates described earlier. Although the *Tetramitus* species other than *T. rostratus* are less well described, it seems that the general trend in this genus is toward loss of the amoeba stage, rather than loss of the flagellate stage which has occurred in some isolates of *T. ampliporus*.

In the present conception of the Vahlkampfiidae, with three monotypic genera (*Adelphamoeba*, *Paratetramitus* and *Heteramoeba*), separate generic status may be warranted for this species. Its eventual position in the family will depend on further studies of its relationship to other *Tetramitus* species and to *Naegleria*, and on hypotheses concerning phylogenetic trends in the family (trends, for example, in flagellar number, reduction/loss of the cytostome, aquisition of pores in the cyst wall).

3.3.3 *Vahlkampfia*

The name *Vahlkampfia* was proposed by Chatton and Lalung-Bonnaire (1912) for the amoeba in which promitosis was first observed by Vahlkampf (1905). Calkins (1913) emended the genus to exclude amoebae able to transform to flagellates, for which he retained the name *Naegleria* which had been proposed for the same group of organisms.

Singh (1952) rejected the name *Vahlkampfia* in the belief, shared by some other authors (e.g. Fulton, 1970), that Vahlkampf had studied *Naegleria gruberi*. However this is not certain, because the cyst illustrated by Vahlkampf (and reproduced by Page, 1976) does not resemble a *Naegleria* species. Since Vahlkampf's culture did not survive for study by others the name cannot follow a type, and Calkins' emendation remains valid. The name *Schizopyrenus* proposed by Singh (1952) to replace *Vahlkampfia* is considered here to be a junior synonym.

Most of the *Vahlkampfia* species isolated could be assigned to one of six species described by Singh (1952) or Page (1967a, 1974). Many of the older descriptions have been reviewed by Page, and illustrations and diagnostic characters reproduced in his 'Illustrated Key' (Page, 1976). None of these species was recognized in the course of this study.

Vahlkampfia inornata and *Paratetramitus jugosus* were the most common Vahlkampfiids, apart from *Naegleria* species, isolated in this study. *V. russelli* (Figures 3.22, 3.23), *V. ustiana* (Figure 3.24) and *V. aberdonica* were less common, while *V. atopa* (Figure 3.25) and *V. avara*, although fairly easily recognized, were rarely isolated.

A previously undescribed species of *Vahlkampfia* was isolated during this study. Like *Tetramitus ampliporus*, it has pores in the walls of its cysts, and could be mistaken for a *Naegleria* species in any study of the distribution of that genus.

The only previous description of a *Vahlkampfia* species with pores in the cyst is that of *V. baltica* Schmoller, 1961, which does not

match either of the species described here. Schmoller's illustrations suggest that his description was based on a mixed culture. The cysts appear to belong to *Acanthamoeba polyphaga*, while the trophozoites look more like a *Saccamoeba* species than a Vahlkampfiid. Intranuclear mitosis was not demonstrated.

Sampling data for the samples from which the new amoeba was isolated are presented in Table 3.2. Dimensions cited in the diagnosis were based on measurements of 50 individuals from plate cultures grown with live *E. coli*. Tests for transformation to flagellates and measurements of trophozoites were made using young (48 hour) cultures. Trophozoites from cultures of the same age were stained by the Feulgen technique for demonstration of promitosis. Measurements of cysts were made using older cultures. A discussion of the taxonomic significance of the new species follows the diagnosis.

Vahlkampfia angularis n.sp. (Figures 3.26-3.30)

type strain : SWL 8994/20

type location : Happy Valley Reservoir, Sth. Australia, 4.7.77

no other isolate known

Diagnosis

Trophozoite usually mononucleate, length in locomotion 21 to 50 µm (mean 31 µm) with mean length/breadth ratio 2.5. Locomotion by slowly eruptive hemispherical bulges from anterior ectoplasm. Usually a single discharging contractile vacuole formed by coalescence of several smaller vacuoles. Uroid consisting of a broad, filamentous margin posteriorly, sometimes present as a broad, thin, frayed sheet.

Nucleus 3.0 to 8.3 µm diameter (mean 6.2 µm) with a large central nucleolus; sometimes surrounded by irregular, large granules, rarely in a complete ring. Division by intranuclear mitosis without interzonal bodies. Temporary flagellate stage not observed.

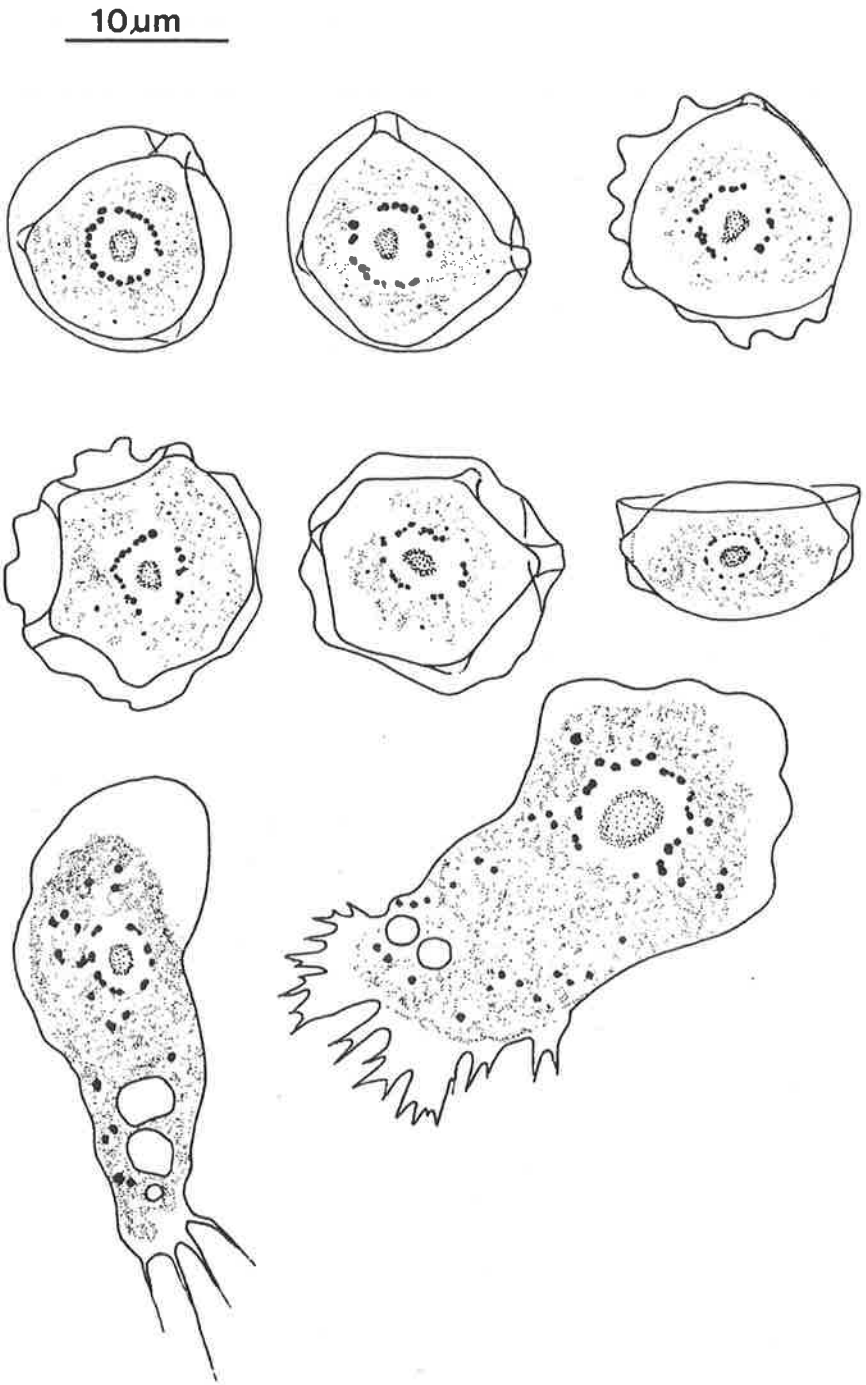


Figure 3.26 ***Vahlkampfia angularis*** n. sp.

Figure 3.27
Vahlkampfia angularis, n. sp. 8994/20, trophozoite

Figure 3.28
V.angularis 8994/20 encystment

Figure 3.29
V.angularis 8994/20 cyst

Figure 3.30
V.angularis 8994/20 cysts

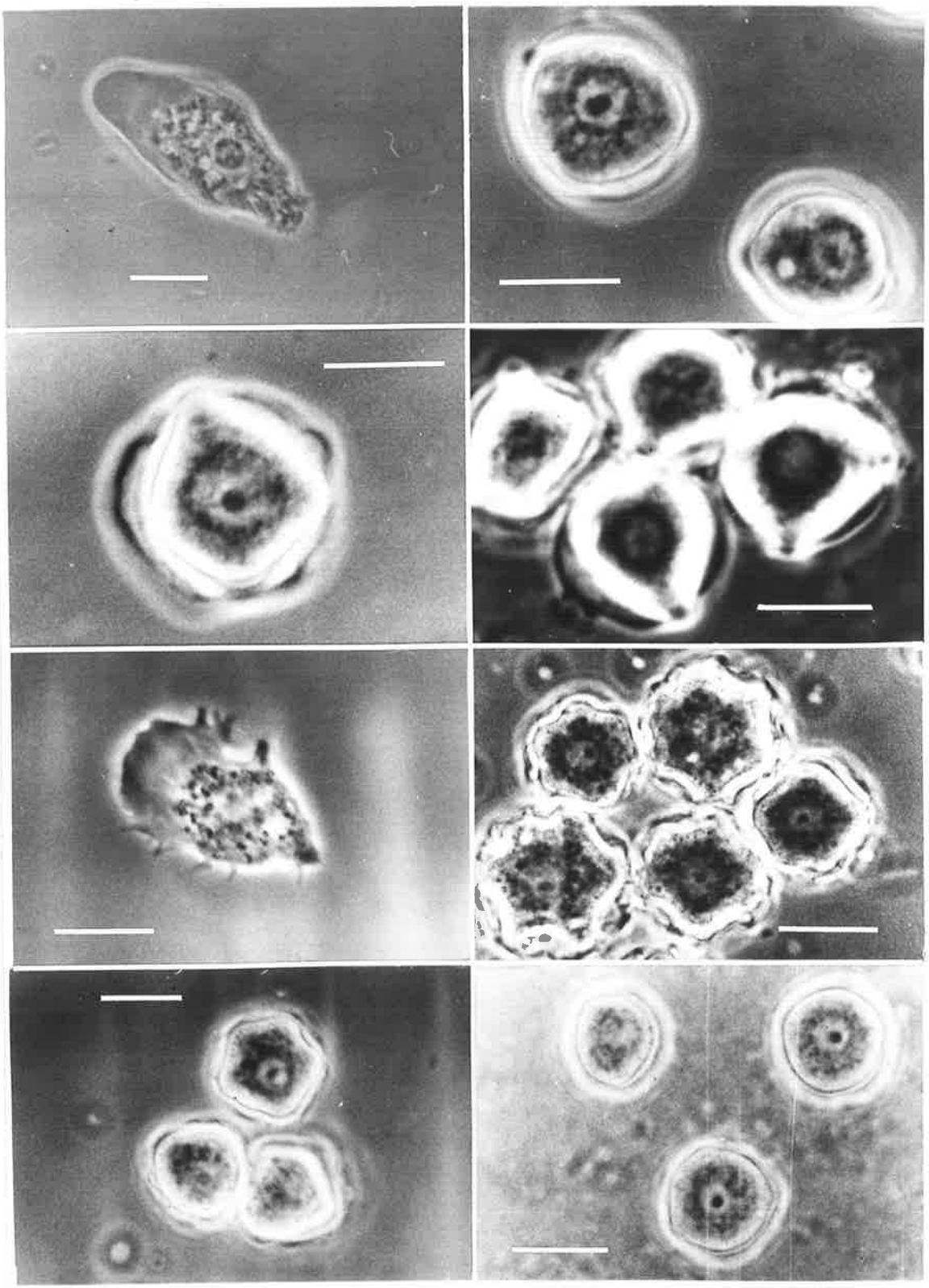
Figure 3.31
Acanthamoeba castellanii 11905, trophozoite

Figure 3.32
A.castellanii 12001/37 cysts

Figure 3.33
A.polyphaga 12444/28 cysts

Figure 3.34
A.palestinensis 10105/37 cysts

Scale = 10 µm



Cyst usually mononucleate, maximum dimension 11.8 to 18.8 µm (mean 15.5 µm). Ectocyst approximately circular, smooth or irregularly rippled; endocyst angular or a rounded polygon. Excystment via one of several pores (1 to 5, mean 2.7) situated in one plane at angles of endocyst. Ectocyst usually not closely applied to endocyst, even at pores. Pores cylindrical, depth often equal to or greater than diameter (may approach 2.0 µm), thickly plugged. No plug or operculum observed inside vacant cyst-wall following excystment.

Discussion

Some cysts of *Vahlkampfia angularis* bear a superficial resemblance to those of certain *Acanthamoeba* strains, and initial examination of trophozoites and cysts suggested a mixed culture. However, the deep pores are unlike those of any described *Acanthamoeba* species, although there is some resemblance to the cysts of 'rough' strains of *Naegleria gruberi* (Figure 3.7). The ectocyst of *V. angularis* projects asymmetrically into a rim, formed against the substrate and parallel to the plane of the pores (Figure 3.26). It resembles the 'brim' described in the cysts of *Adelphamoeba galeacystis* (Napolitano *et al.*, 1970). However, a similar asymmetry has been observed in cysts of *Vahlkampfia russelli* and *Paratetramitus jugosus* isolated in this study. It appears to result simply from encystment against a surface (perhaps in any species in which endocyst and ectocyst are not closely attached) and it is not accorded any taxonomic significance here.

Under conditions which stimulate transformation to flagellates in *Naegleria* species, most trophozoites of *V. angularis* began to encyst. Encystment was rapid and nearly synchronous; within two hours at 28°C, the ectocyst was forming and the eventual positions of the pores were recognisable (Figure 3.28).

V. angularis is known only from the type strain, which is unable to grow above approximately 30°C. While it is apparently extremely uncommon, it is important that it be distinguished from *Naegleria* species should it be isolated in a study such as that presented here.

3.4 *ACANTHAMOEBA*

The correct generic name of the amoebae placed here in the genus *Acanthamoeba* is another point of disagreement between Singh (1975) and Page (1976).

Singh (1952) rejected the form of the cyst wall as an important generic criterion, accepting an older, more inclusive definition of the genus *Hartmannella* based solely on the mode of nuclear division. However, the amoebae which would be assigned to *Hartmannella* under Singh's definition '... have nothing in common ... except a general mitotic pattern which they share ... with metazoan cells' (Page, 1967b). A number of amoebae, not considered by Singh (e.g. *Vannella* spp.), which are even less like the original description of *Hartmannella*, would be included.

Page (1967b), in redefining *Acanthamoeba*, showed that it is a group of several distinguishable amoebae, united by producing double-walled cysts with a mammilate outer wall (Figures 3.32 to 3.34) and by their form in locomotion. *Acanthamoeba* derives its name from the numerous 'acanthopodia' produced from the edge of the broad 'lobopodium' (Figure 3.31).

Other authors have shown biochemical similarities between *Acanthamoeba* strains (Adam, 1964), and serological distinctness from *Hartmannella* species with some cross-reaction between *Acanthamoeba* strains (Visvesvara and Balamuth, 1975; Willaert and Stevens, 1975). Page's nomenclature is accepted by authors using *Acanthamoeba* for studies

on the mechanism of encystment as a model of differentiation (Weismann, 1976).

In this study several described species of *Acanthamoeba* could be distinguished by the structure of the cyst and the relative breadth of the amoeba in locomotion. *Acanthamoeba* isolates corresponding morphologically to *A. castellanii*, *A. polyphaga*, *A. palestinensis* (Figures 3.31 to 3.34), *A. lenticulata* and *A. hatchetti* were identified. *A. astronyxis* and *A. royreba*, which have quite distinctive cysts, were not encountered among the several hundred *Acanthamoeba* isolates identified. *A. palestinensis* and *A. culbertsoni* could not be distinguished morphologically as claimed by Visvesvara and Balamuth (1975). The type strain of *A. culbertsoni* has a high temperature tolerance, growing at 42°C to 43°C (Griffin, 1972). Temperature tolerance of *Acanthamoeba* species isolated is discussed in Section 4.2.

A. rhysodes is considered here to be a synonym of *A. castellanii*, following Page. Validity of the morphological species and synonymy recognized by Page (1967b, 1976) have largely been confirmed by the serological studies (Visvesvara and Balamuth, 1975; Willaert and Stevens, 1976). Unfortunately, antisera have not been raised against all type and reference strains; those which have been prepared are not available in Australia.

3.5 OTHER AMOEBAE

The larger amoebae, which usually feed on algae or smaller protozoa, were not commonly isolated by the methods used. However, occasional isolates of *Polychaos* species (Figure 3.35), *Mayorella* species (Figure 3.36), and *Thecamoeba* species (Figure 3.37) were observed when suitable food organisms were present in the sample.

Many smaller bacteria-feeding amoebae, which have no known public health significance, could be identified from descriptions and

Figure 3.35
Polychaos sp. 13174/28
trophozoite

Figure 3.36
Mayorella sp. 13073/28
trophozoite

Figure 3.37
Hartmannella vermiformis
6967/28, trophozoites

Figure 3.38
Saccamoeba sp. 13073/28
trophozoite

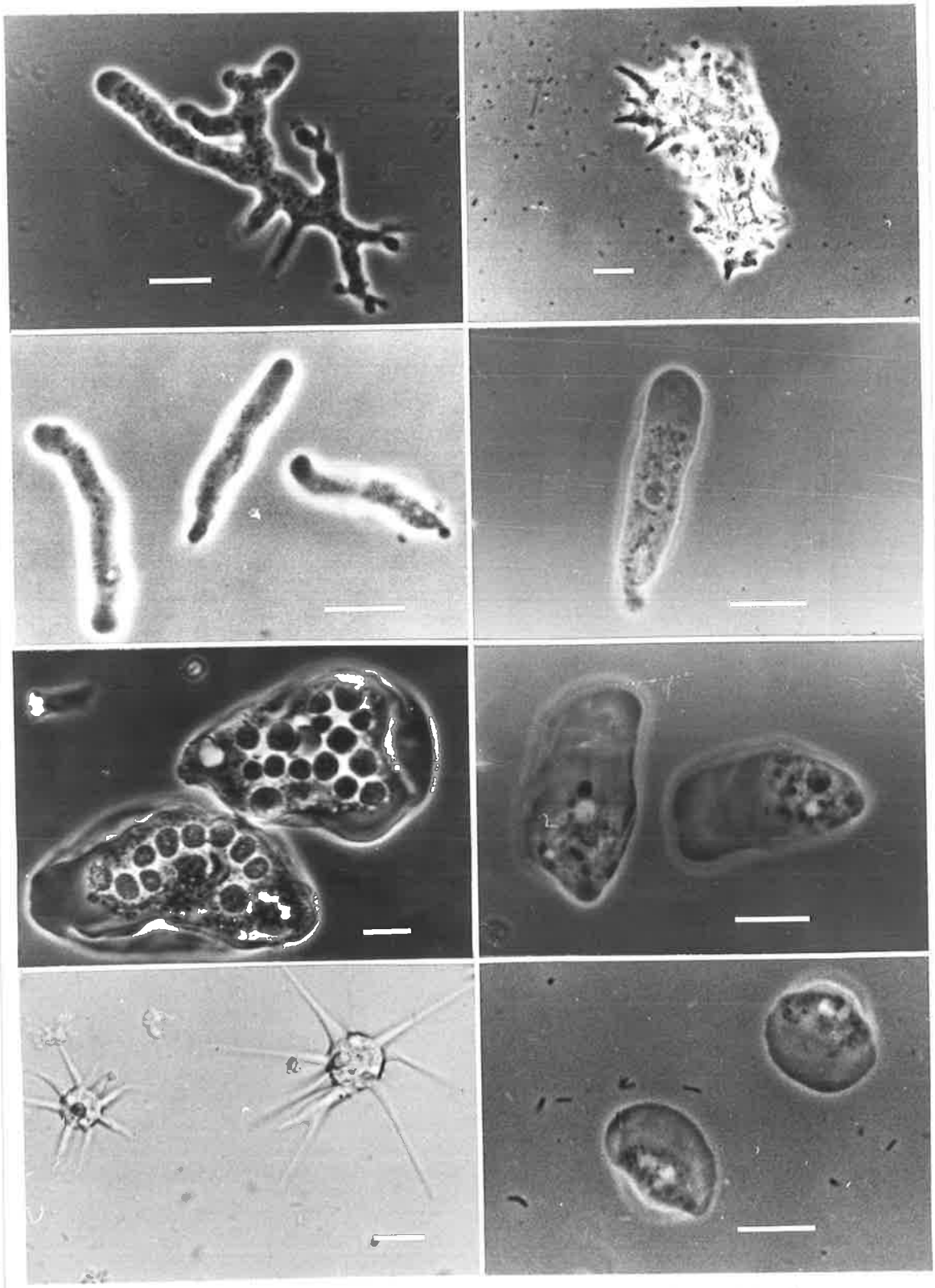
Figure 3.39
Thecamoeba terricola
Pk-130/28, trophozoite

Figure 3.40
Platyamoeba placida
13457/28, trophozoites

Figure 3.41
Vannella mira 602/28
floating form

Figure 3.42
Vannella mira 13723/28
locomotive form

Scale = 10 µm



illustrations, in particular from Page's 'Illustrated Key' (Page, 1976), and are listed in Table 3.1. The distribution records cited by Page and a search of the generic index of the Zoological Record from 1950 forward indicate that only *Naegleria* and *Acanthamoeba* species have been formally reported from Australia before.

Two groups seemed potentially useful in tracing the source of contamination of water. For example, swimming pools and storage tanks are susceptible to contamination by soil, including air-borne dust, or by introduction of inadequately disinfected water.

Vannella species were easily recognized by the radiate floating form (Figure 3.41) which settles, usually very slowly, to a broad, fan-shaped locomotive form (Figure 3.42). The strains of *Vannella* discussed in the literature are apparently unable to produce cysts, and have not been isolated from soil (Page, 1968, 1976). Most isolates could be assigned readily to one of three species recognized by Page (*V. platypodia*, *V. mira* and *V. simplex*), and did not produce cysts in culture. From several sample locations *Vannella* species were the most commonly isolated amoebae and most samples contained two or all three species.

Hartmannella vermiformis is a small, elongate amoeba which is able to encyst, and has been isolated from soil and leaf litter as well as freshwater (Page, 1976). In this study, *H. vermiformis* (Figure 3.38) was one of the commonest amoebae in samples of reticulated water.

The distribution of *Hartmannella vermiformis* and *Vannella* species and their usefulness in assessing possible sources of contamination is discussed in Section 5.5.

4. THE INFLUENCE OF TEMPERATURE ON THE BIOLOGY OF AMOEBAE

4.1 INTRODUCTION

The epidemiological link between groups of cases of primary amoebic meningoencephalitis in different countries is contact with unusually warm water, particularly by swimming (Symmers, 1969; Carter, 1972). In warmer parts of the United States of America, infections have followed bathing in natural freshwater lakes (Butt *et al.*, 1968; Duma *et al.*, 1971). However in temperate climates the immediate sources of infection have been rather diverse: in Czechoslovakia, an artifically heated swimming pool (Cerva and Novak, 1968); in Belgium, a stream receiving cooling water from a factory (van den Driessche *et al.*, 1973); in New Zealand, a pool fed by geothermal springs (Nicol, 1973); in Britain, shallow rainwater puddles (Saygi *et al.*, 1973) and a natural spa (Bath Public Health Laboratory, 1978).

Several publications have cited temperature measurements in water implicated in infections by *Naegleria*, or temperatures associated with samples examined for pathogenic amoebae (Table 4.1). Most of the temperature figures relate to a small number of samples collected close together in time. However, Wellings *et al.* (1977) illustrated the seasonal variation in water temperature in freshwater lakes in Florida.

In the European studies water temperature rarely exceeded 30°C, and it has been assumed by several authors that selection for pathogenic amoebae may take place around that temperature. In the American studies temperatures around 35°C were more common, and in a lake receiving a thermal discharge in Texas, *N. fowleri* was isolated when the temperature was 39 - 41°C (Stevens *et al.*, 1977).

In the present study, the temperature of reticulated water in Pt Augusta and Pt Pirie exceeded 30°C at times during each of the five summers between 1974/75 and 1978/79 (Table 4.2). Temperature increases of 3 to 7°C occurred in the trunk main during pumping of

TABLE 4.1. WATER TEMPERATURE ASSOCIATED WITH INFECTIONS OR WITH SAMPLING FOR PATHOGENIC *NAEGLERIA* and *ACANTHAMOEBA*

REFERENCE	LOCATIONS	WATER TEMPERATURE (°C)
Cerva and Novak, 1968	Swimming pool, Czechoslovakia	24
Kadlek *et al.*, 1978	As above	27 - 30
van den Dreissche *et al.*, 1973	Canal and stream (thermal discharge), Belgium	26.5 - 30
de Jonckheere *et al.*, 1975	As above	29 - 30, 34
Willaert *et al.*, 1977	As above	32
Stevens *et al.*, 1977	Lakes (some with thermal discharge), Florida	29 - 38
	Lakes (some with thermal discharge), Texas	29 - 41
Wellings *et al.*, 1977	Lakes (some with thermal discharge), Florida	seasonal range shown, as high as 34.5 - 36 in summer

TABLE 4.2.

HIGHEST RECORDED WATER TEMPERATURES, SOUTH AUSTRALIA 1974 - 1979

YEAR:-	1974/75	1975/76	1976/77	1977/78	1978/79
Location					
R. Murray at Morgan	28	27	30	27	27
Morgan after Cl_2	27	28	28	27	27
Napperby bf Cl_2	31	31.5	31	31	34
Nelshaby Reservoir	29	30	28	29	29.5
Georges Corner	30	30	30	29	33
Pt Pirie-reticulated water	31	32	32	33	35
Stirling North bf Cl_2	32	34	34.5	35	34
Pt Augusta Causeway	30.5	32	32.5	34	32.5
Pt Augusta-reticulated water	35	35	36	35.5	37
Paskeville No. 1 Resv.	28	27.5	25	23	27
Paskeville No. 1 after Cl_2	27	27	25	24	26
Kadina	34	32	24	24	27
Hope Valley Resv.	24	22	25	24	24
Hope Valley after Cl_2 (Lyons Rd)	22.5	24.5	22	27	25

water above ground from the River Murray at Morgan where water temperatures rarely reached 30°C. The temperature of water supplied from Hope Valley, a major water source for metropolitan Adelaide where amoebic meningoencephalitis has not been recorded, rarely exceeded 25°C, even during summer. Seasonal variation in water temperature at several reservoirs is shown in Figures 4.2 to 4.5.

Griffin (1972) demonstrated that nine isolates of *N. fowleri* from human infections would grow at 44° to 45°C, whereas three reference strains of *N. gruberi* grew at 37°C but did not survive higher temperatures. Temperature tolerance is now known for nearly 120 *N. fowleri* isolates from infections or from water or sediment samples (compiled from the literature and unpublished results). All will grow at least at 44°C.

The virulence of known pathogenic strains of *Acanthamoeba* is more variable and human infections have varied in their severity (see Section 1.3). Correspondingly, the temperature tolerance of the pathogens which have been studied is less uniform than that of *N. fowleri*, and is less distinct from related avirulent strains. Griffin (1972) showed that the highly virulent pathogen *A. culbertsoni* grew at 42 - 43°C, while *Acanthamoeba* strains of lower virulence to mice (assigned to '*A. rhysodes*') could not grow above 37 - 39°C. The temperature tolerance of four nonpathogenic reference strains of *Acanthamoeba* varied from 34°C to 37°C.

4.2 DISTRIBUTION OF TEMPERATURE TOLERANCE AMONG *NAEGLERIA* AND *ACANTHAMOEBA* ISOLATES

The temperature tolerance of a large number of *Naegleria* and *Acanthamoeba* isolates from the field sampling was determined from their growth with *E. coli* on a solid medium (Section 2.7). The method specified the upper limit of temperature tolerance to within 2°C, but

did not provide a lower limit. A lower temperature limit for growth was determined for a few strains in the more detailed growth experiments described in Section 4.3.

The frequency distribution of temperature tolerance of all *Naegleria* and *Acanthamoeba* isolates tested is shown in Figure 4.1.

For *Naegleria* species, the lowest tolerance was shown by a few isolates which grew at 30°C but not at 33°C. At the other end of the distribution, a small number of isolates was able to grow at 44°C. A few of these were identified as *Naegleria fowleri* by their pathogenicity for laboratory animals; other, nonpathogenic strains probably represent the recently described *N. lovaniensis*, although characters other than pathogenicity and temperature tolerance have yet to be examined (see Section 3.3.1). The identity of many *Naegleria* isolates able to grow at 42°C but not at 44°C warrants further attention. It may be that *N. lovaniensis* has a less uniform temperature tolerance than is suggested from the few isolates which have been examined (Stevens *et al.*, 1980). Isolates identified as *N. gruberi*, either from their agglutination titres or from the structure and number of pores in their cysts, varied from those that did not grow above 30°C to a few isolates able to grow at 42°C but not at 44°C.

Temperature tolerance of *Acanthamoeba* isolates was far less varied than that of the *Naegleria* species ; 91 percent grew at 37°C or above, but only 5 per cent could grow at 42°C. *Acanthamoeba* isolates which could grow above 40°C commonly had the '*palestinensis/culbertsoni*' cyst morphology (see Section 3.4). There were no observable differences between temperature tolerances of the other morphologically distinguishable *Acanthamoeba* species.

The seasonal distribution of temperature tolerance of *Naegleria* species isolated from water samples collected weekly at several reservoirs in South Australia is shown in Figures 4.2 to 4.5. For some samples,

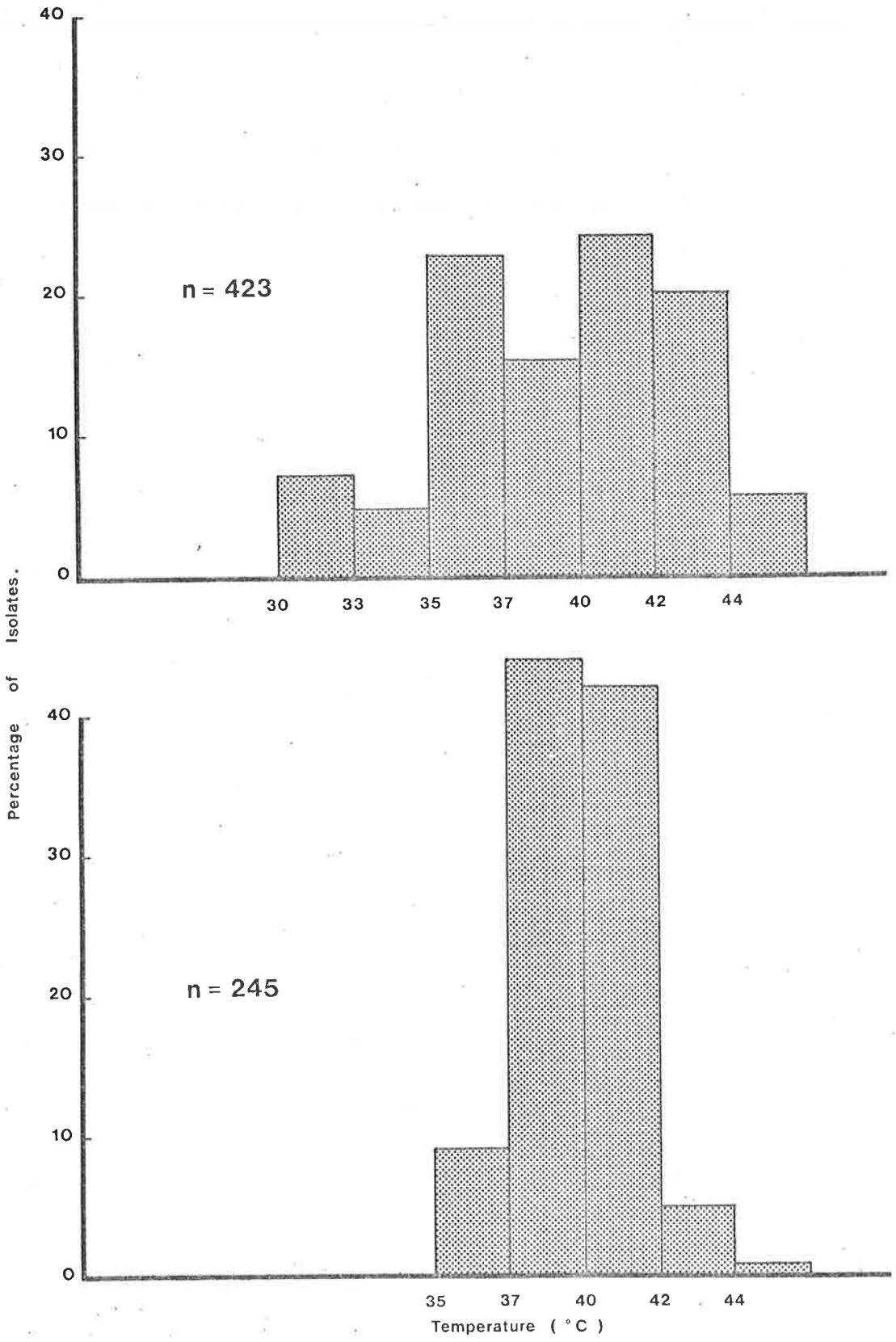


Figure 4.1 . Frequency distribution of temperature tolerance of *Naegleria* . (above) and *Acanthamoeba* (below) isolates from field samples .

Naegleria strains with different temperature tolerances were isolated at different incubation temperatures. For simplicity, only the highest tolerance from any sample is illustrated. Calculation of correlation coefficients was also based on the isolate with the highest temperature tolerance in any sample.

The temperature tolerance of *Naegleria* species from Nelshaby Reservoir during 1978/79 (Figure 4.2) correlated significantly with water temperature at the time of sampling (correlation coefficient, $r = 0.6608$, $P < 0.001$). Temperature tolerance of *Naegleria* isolates from Paskeville No. 1 Reservoir (Figure 4.3) did not correlate significantly with sample temperature ($r = 0.4378$), although correlation with the three-week running mean of sample temperatures was significant ($r = 0.5410$, $P < 0.05$). Isolation of 40°C- and 42°C-tolerant strains during and following some unseasonally warm weather in June (Figure 4.3) seems to have been responsible for the lower significance level. The warm weather was not strongly reflected in the sample temperatures, suggesting that the weekly sampling frequency made the survey insensitive to some short-term changes.

Correlation of temperature tolerance of *Naegleria* isolates from Hope Valley Reservoir with water temperature during 1978/79 (Figure 4.4) was highly significant ($r = 0.7021$, $P < 0.001$).

N. fowleri was not isolated from any of these water sources during 1978/79. However, two isolations of *N. fowleri*, confirmed by their pathogenicity to laboratory animals, were made from Paskeville No. 1 Reservoir during the 1979/80 summer (Figure 4.5, $r = 0.5295$, $P < 0.001$).

While pathogenic *Naegleria* were not isolated in 1978/79, many summer isolates from the water sources in areas where infections by *N. fowleri* have occurred were capable of growth at 42°C (Figures 4.2, 4.3). At Hope Valley Reservoir, however, 42°C-tolerant *Naegleria*

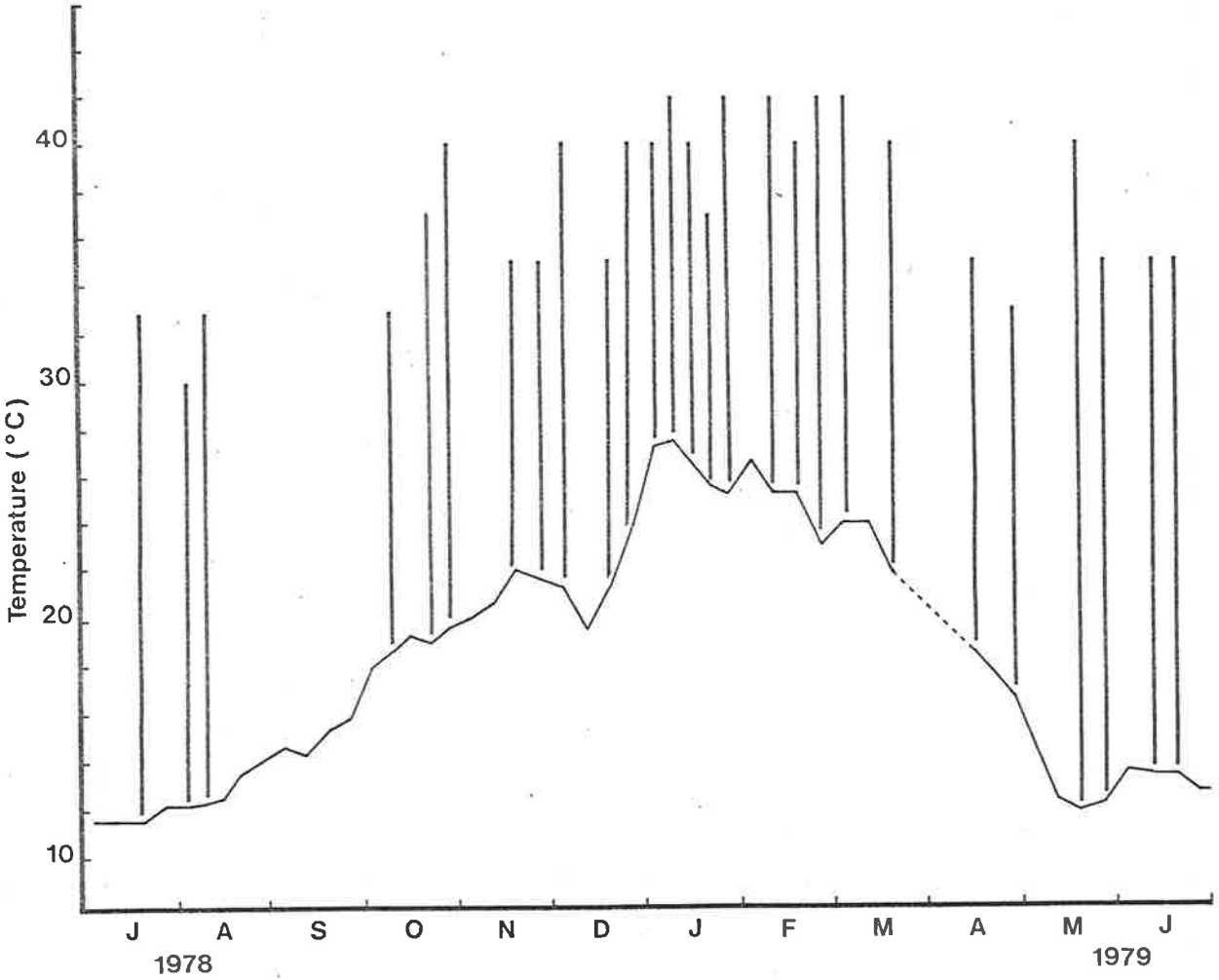


FIGURE 4.2 SEASONAL VARIATION IN WATER TEMPERATURE AND TEMPERATURE TOLERANCE OF *Naegleria* ISOLATED FROM NELSHABY RESERVOIR, 1978/79.

| RANGE OF TEMPERATURES OVER WHICH GROWTH OF *Naegleria* OCCURRED, TRUNCATED BELOW AT WATER TEMPERATURE

——— WATER TEMPERATURE AT TIME OF SAMPLING (THREE-WEEK RUNNING MEAN).

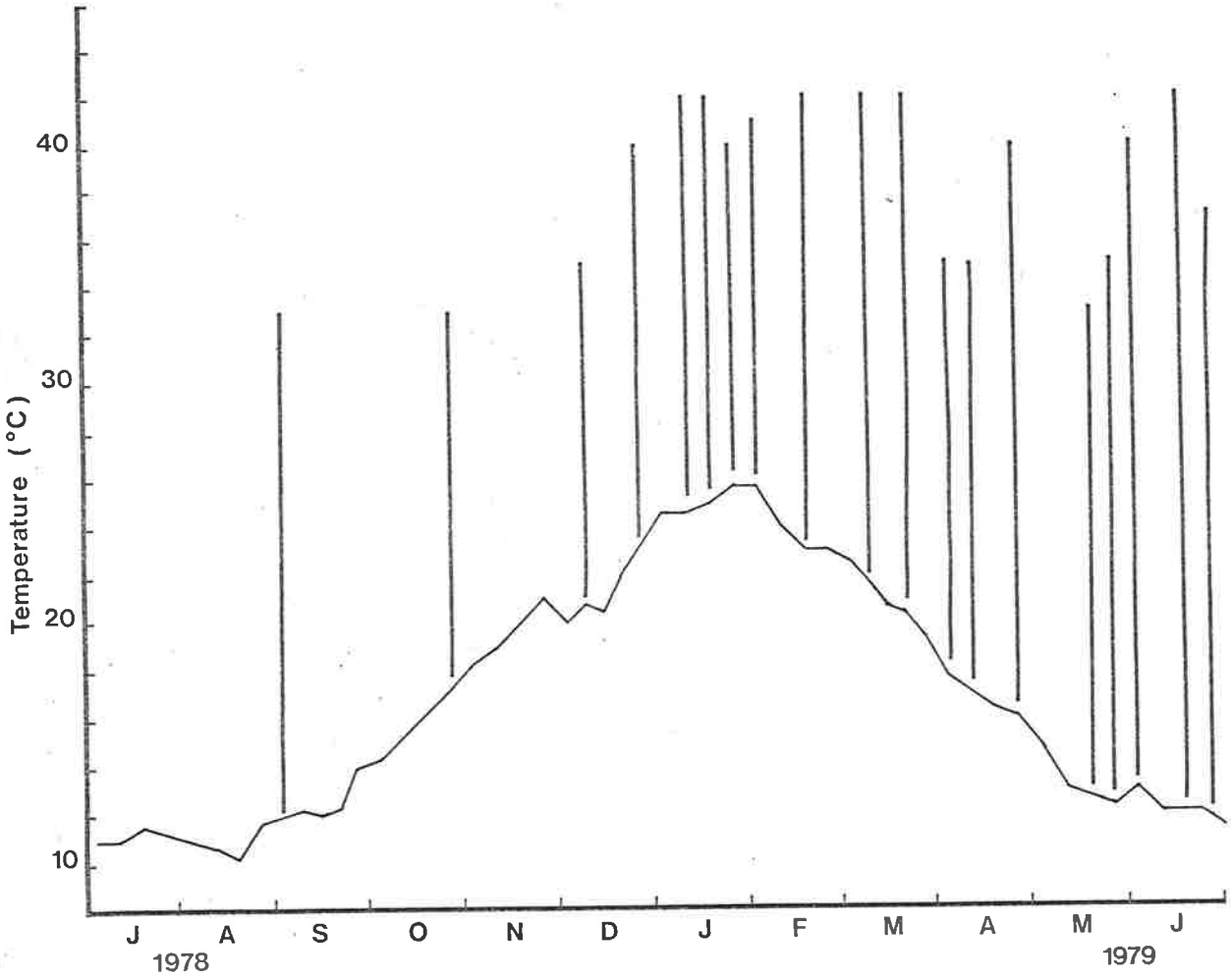


FIGURE 4.3 SEASONAL VARIATION IN WATER TEMPERATURE AND TEMPERATURE TOLERANCE OF *Naegleria* ISOLATED FROM PASKEVILLE No.1 RESERVOIR, 1978/79.

| RANGE OF TEMPERATURES OVER WHICH GROWTH OF *Naegleria* OCCURRED, TRUNCATED BELOW AT WATER TEMPERATURE

——— WATER TEMPERATURE AT TIME OF SAMPLING (THREE-WEEK RUNNING MEAN).

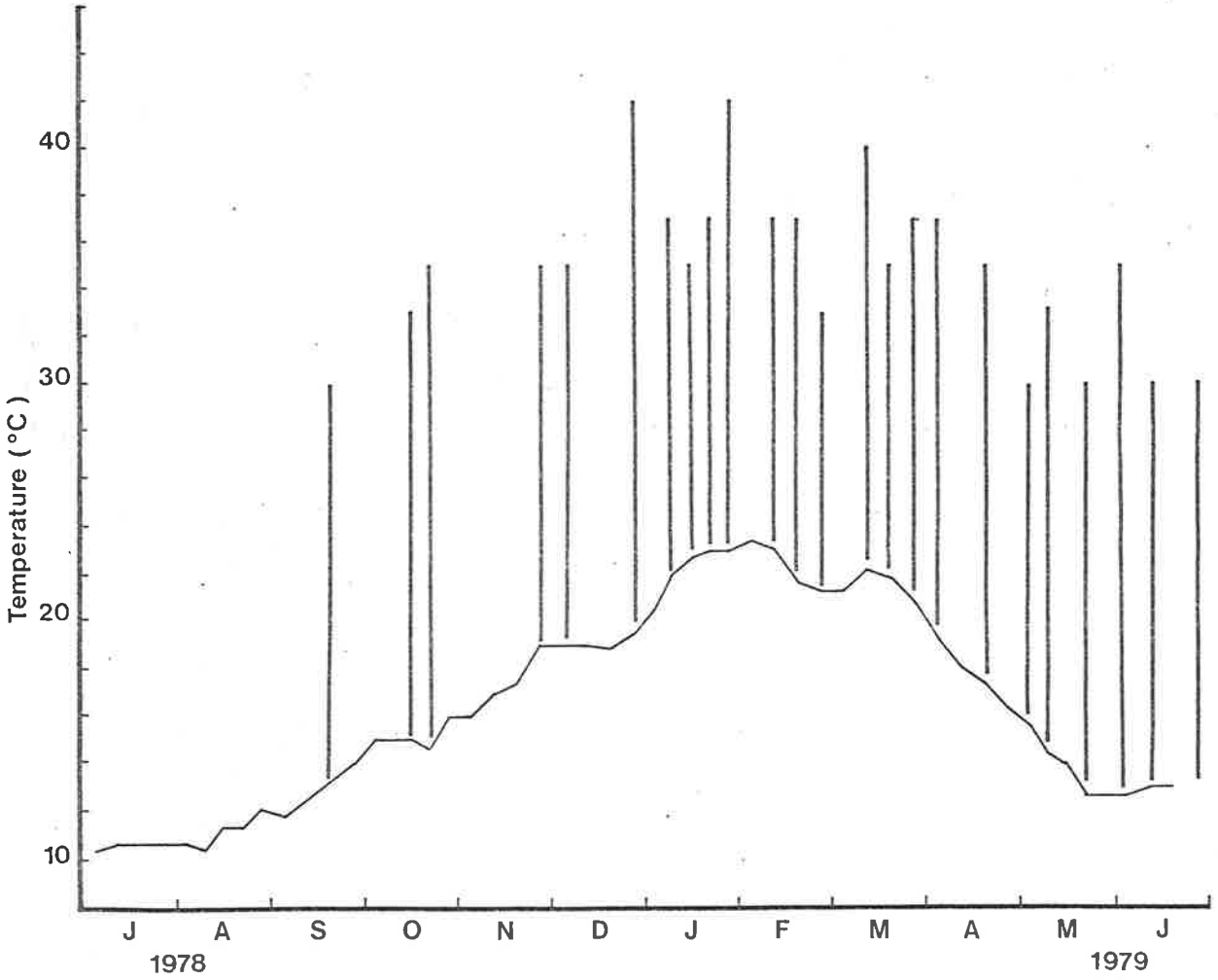


FIGURE 4.4 SEASONAL VARIATION IN WATER TEMPERATURE AND TEMPERATURE TOLERANCE OF *Naegleria* ISOLATED FROM HOPE VALLEY RESERVOIR, 1978/79.

| RANGE OF TEMPERATURES OVER WHICH GROWTH OF *Naegleria* OCCURRED, TRUNCATED BELOW AT WATER TEMPERATURE

——— WATER TEMPERATURE AT TIME OF SAMPLING (THREE-WEEK RUNNING MEAN).

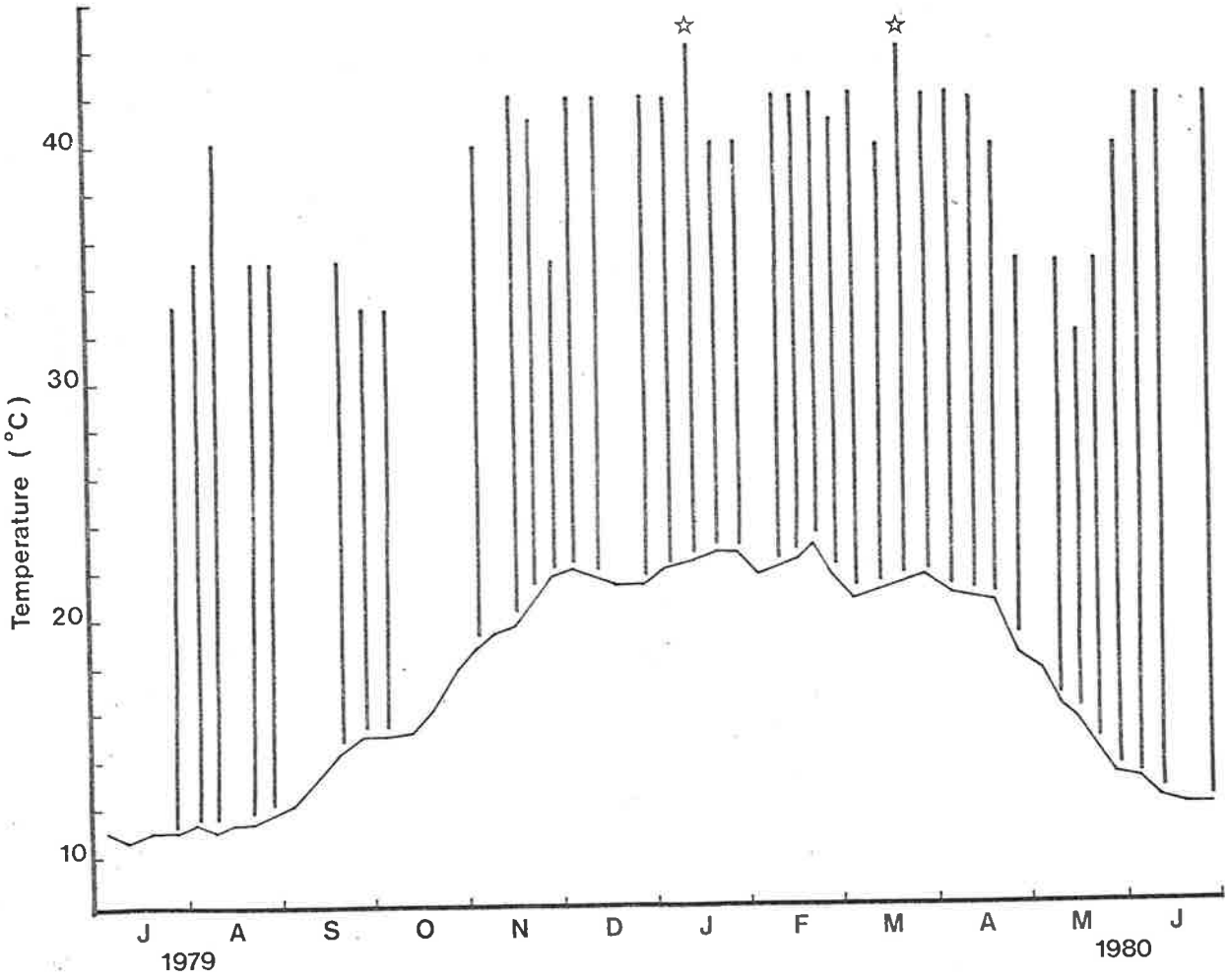


FIGURE 4.5 SEASONAL VARIATION IN WATER TEMPERATURE AND TEMPERATURE TOLERANCE OF *Naegleria* ISOLATED FROM PASKEVILLE No.1 RESERVOIR, 1979/80

☆ *Naegleria fowleri*

| RANGE OF TEMPERATURES OVER WHICH GROWTH OF *Naegleria* OCCURRED, TRUNCATED BELOW AT WATER TEMPERATURE

——— WATER TEMPERATURE AT TIME OF SAMPLING (THREE-WEEK RUNNING MEAN)

were isolated from only two samples; the majority of summer isolates did not grow above 37°C (Figure 4.4).

The seasonal variation in temperature tolerance of *Naegleria* isolates presumably reflects seasonal changes in abundance of different *Naegleria* strains. Isolation of *N. fowleri* clearly depends on the relationship between its absolute abundance and the volume of the sample collected. Important objectives of future field surveys should be development of quantitative methods for examining water samples, choice of appropriate sample volumes and estimation of densities of *Naegleria* species (particularly of *N. fowleri*) in water and bottom sediments.

4.3 INFLUENCE OF TEMPERATURE ON GROWTH RATES OF *NAEGLERIA* SPECIES

The seasonal distribution of *Naegleria* species cannot be explained simply in terms of the upper limits of temperature tolerance of different strains. Summer water temperatures at Nelshaby and Paskeville No. 1 Reservoir were rarely high enough to be lethal even to trophozoites of *Naegleria gruberi* strains with the lowest temperature tolerances. Many nonpathogenic strains could grow even at the highest temperatures reached in reticulated water in Pt Augusta and Pt Pirie. The apparent fall in density of 'high temperature tolerance' strains during cooler months also needs more explicit explanation.

Several *Naegleria* strains, including *N. fowleri* isolates from human infections and from freshwater, were selected for an experimental study of the influence of temperature on growth rates. The word 'growth' is used here in the sense of 'growth of cell numbers'. Although it could be considered ambiguous - growth of cell volume rather than multiplication could be inferred - it is preferred because terms such as 'growth rate constant' and 'temperature coefficient of growth' are widely used for microorganisms to refer to growth of cell numbers.

The method used to assess growth and the derivation of growth rate constants, temperature coefficients and generation times are explained in Section 2.8.

The influence of temperature on growth rate of a reference strain of *N. gruberi* (CCAP 1518/1c) growing under different culture conditions is shown in Figure 4.6. Growth rates in Fulton's Medium A (CFA) in the first and sixth subculture following prolonged cultivation with *E. coli* are shown. In each case, growth rate constant appeared to be a linear function of temperature from the lower limit of temperature tolerance ($k_{24} = 0$) to within $2^{o}C$ of the temperature optimum. Above the temperature optimum, the growth rate fell rapidly until the upper limit of temperature tolerance (where $k_{24} = 0$ again) was reached.

Absolute growth rates at any temperature differed between the experiments in a manner that suggested that gradual adaptation to the medium occurred. Regression lines fitted to each experiment intersected the line $k_{24} = 0$ very close together.

In another experiment, single trophozoites of *N. gruberi* CCAP 1518/1c were used to initiate clones on agar plates spread with heat-killed *E. coli*. Growth of a clone does not fulfil the assumption of asynchrony implicit in the derivation of the growth rate constant (Section 2.8): estimates of generation time will vary depending on the point in the (synchronous) cell cycle at which the cells are counted. Nevertheless, a line fitted to growth rate constants based on six replicate clones at each temperature intersected the line $k_{24} = 0$ close to the lines for growth in CFA. Confidence intervals for k_{24} values calculated for clones were broader than those for growth in CFA because the replicate clones were not necessarily in synchrony with each other.

Confidence limits for the regression lines were difficult to calculate. Since k_{24} is a logarithmic transformation of a normally

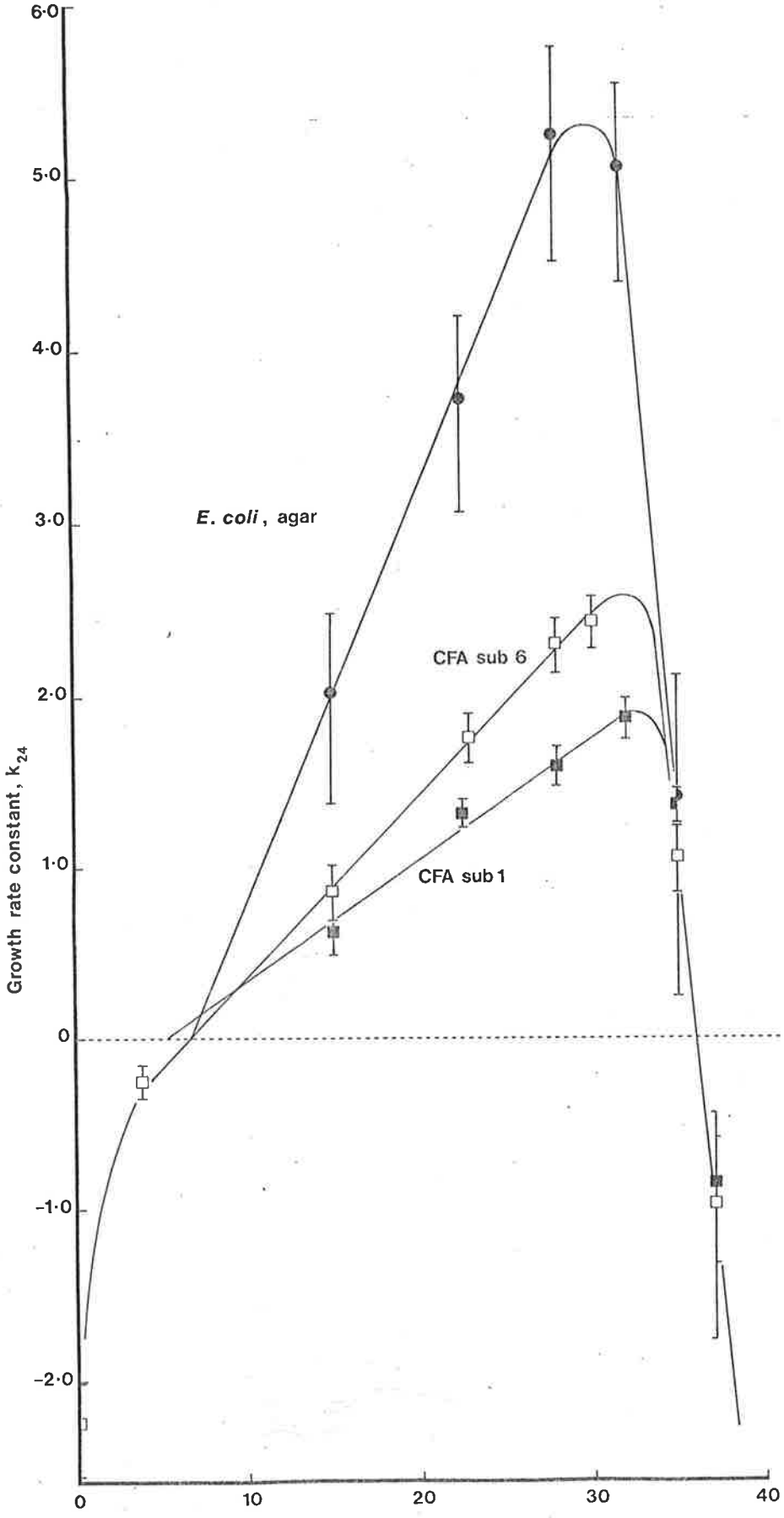


Figure 4.6 Influence of temperature on rate constants (k_{24}) for growth of *Naegleria gruberi* under different conditions. Mean, 95% confidence limits.

distributed quantity (density of organisms), confidence limits at each temperature are asymmetrical and the replicates cannot be entered in the regression analysis. Although it is difficult to demonstrate statistically, it is inferred from Figure 4.6 that the lower limit temperature tolerance of *N. gruberi* is independent of the culture conditions. If this inference is correct, growth of *N. gruberi* over most of its temperature range can be described by a line of the form

$$k_{24} = a\,(T - T_L) \qquad \text{(Eq. 4.1)}$$

where T is the growth temperature, T_L is the lower limit of temperature tolerance and a is a variable describing the influence of other conditions (e.g. bacterial density) on growth rate; provided, of course, that the density of *Naegleria* trophozoites permits log-phase growth.

For the linear relationship of k_{24} to temperature, the temperature coefficient, Q_{10}, varies continuously with the temperature interval chosen and, for a given interval, is specified by the x-intercept. If the lower limit of temperature tolerance is independent of culture conditions, it follows that Q_{10} for any temperature interval must also be independent of those conditions.

This corollary is important, since it validates the use of temperature coefficients derived for growth in CFA to predict the influence of temperature on growth of *N. gruberi* in a natural fresh water body, even though the absolute growth rate may vary with other conditions such as the bacterial density. Because log-phase growth occurs over a wide range of densities in CFA, Q_{10} can be determined quite accurately.

Figure 4.7 illustrates the influence of temperature on growth rates of several isolates of *N. fowleri* in CFA. *N. fowleri* NORTHCOTT and DAMIANO were isolated from fatal human infections (see Section 2.1).

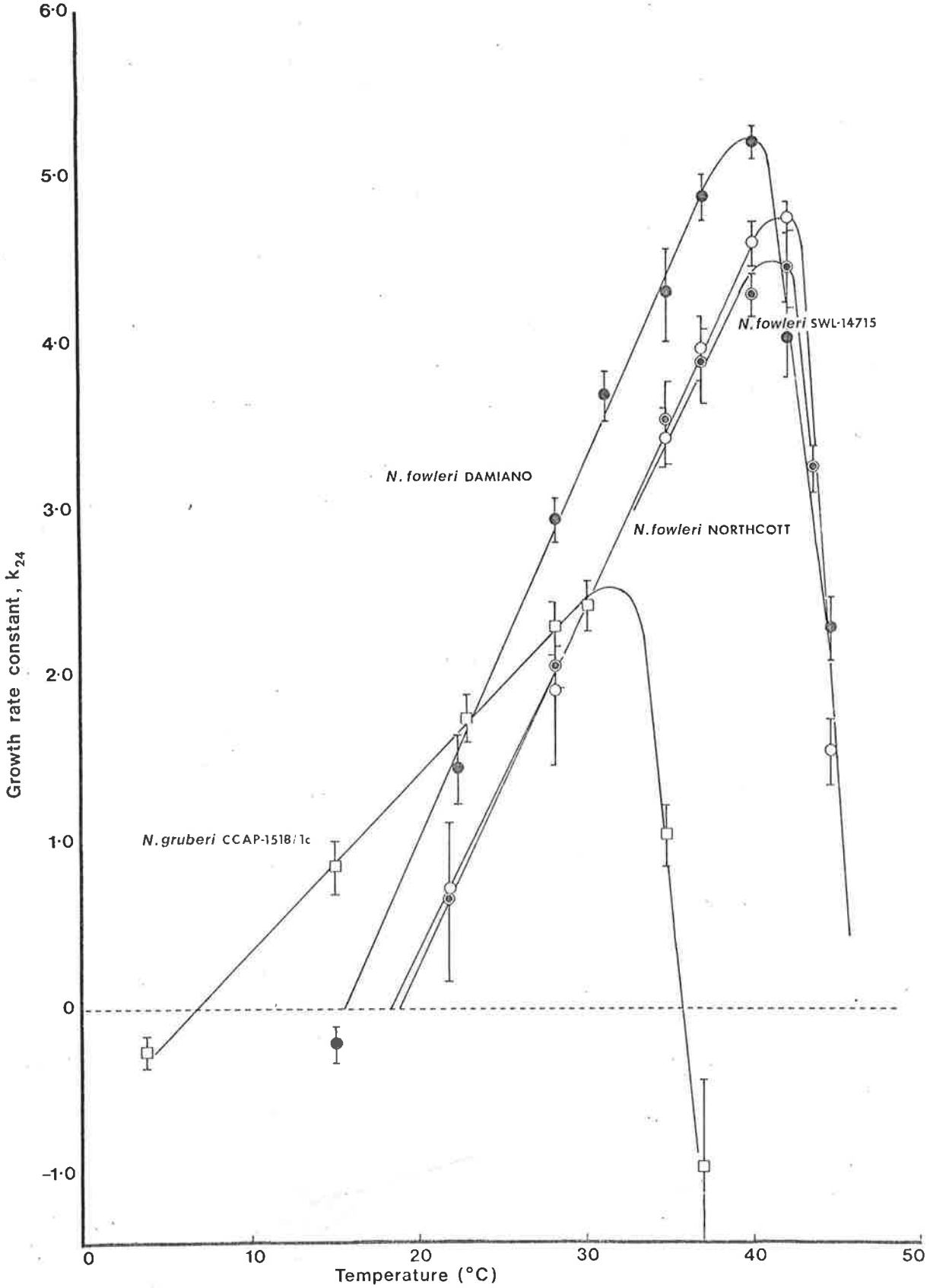


Figure 4.7 Influence of temperature on growth rate constants (k_{24}) of *Naegleria* spp. growing axenically in Fulton's medium A. Mean, 95% confidence limits.

The NORTHCOTT strain has been in culture since 1971, while DAMIANO was the most recent available isolate of human origin. *N. fowleri* SWL 14715 was isolated from a water sample collected in the Paskeville area. Growth of *N. gruberi* in CFA ('subculture 6' from Figure 4.6) is included in the figure for comparison.

The growth curves for *N. fowleri* were similar to those for *N. gruberi* in several respects. Growth rate constant was a linear function of temperature from the lower limit of tolerance to within 2°C of the temperature optimum, which was approximately 3° to 4°C below the upper limit of tolerance.

The similarity suggests that temperature optimum, temperature limits and temperature coefficient of growth for *N. fowleri* are independent of other growth conditions, as they are for *N. gruberi*.

Growth curves for *Naegleria* species differ from those reported for *Trypanosoma* and *Leishmania* species. Chang and Negherbon (1947) reported experiments with these obligate parasites in which growth rate constant was an exponential function of temperature, giving a constant temperature coefficient, over part of the growth range. However, variation of Q_{10} with temperature range is more common for physiological processes in general (Giese, 1962).

Growth statistics for *N. gruberi* and *N. fowleri* estimated from the growth curves (calculated from the regression analysis, in the case of the lower limit of temperature tolerance) are listed in Table 4.3. Growth curves for *N. fowleri* NORTHCOTT and SWL 14715 were almost identical, with optima close to 42°C and upper limits of tolerance approximately 46°C. *N. fowleri* DAMIANO had a similar upper limit of tolerance but a slightly lower optimum (approximately 40°C) and lower limit of tolerance. Temperatures below 16° to 19°C (varying between isolates) were lethal to trophozoites of *N. fowleri*. Water temperatures in all the water bodies sampled in this study, including Paskeville No. 1

TABLE 4.3. GROWTH STATISTICS OF *NAEGLERIA* SPECIES, ESTIMATED FROM GROWTH IN FULTON'S MEDIUM A (FIGURE 4.7)

Strain	Temperature limit (^{o}C) lower	upper	*Q_{10} (20^{o} to 30^{o}C)	Temperature optimum (^{o}C)	*g at Temp. optimum (hr)
N. fowleri					
NORTHCOTT	18.9	46	9.8	42	5.0
DAMIANO	15.7	46	3.3	40	4.6
SWL 14715/37	18.4	46	7.2	42	5.3
N. gruberi					
CCAP 1518/1c	6.6	36	1.7	32	9.4

* Q_{10} = temperature coefficient, g = mean generation time. See Section 2.8 for definitions and derivations.

Lower temperature limit and Q_{10} calculated from regression analysis of linear portion of growth curve.

Reservoir from which *N. fowleri* was isolated in January and March 1980, fell below 16°C for several months each year (Figures 4.2 to 4.5). This confirms the suggestion of Wellings *et al.* (1977) that *N. fowleri* probably survives winter as cysts in bottom sediments. Chang (1978) published evidence that *N. fowleri* cysts are relatively resistant to low temperatures; survival was higher as temperature fell from 15° to 0°C, as long as freezing did not occur. However, absolute values for survival could not be calculated from his figures.

In contrast, the reference strain of *N. gruberi* studied here survived temperatures as low as 7°C, indicating that *N. gruberi* may grow throughout the year in the water bodies for which temperatures are presented in this study.

Temperature coefficients for growth of *N. fowleri* and *N. gruberi* were calculated for the interval 20° to 30°C, the most convenient 10°C range for which the linear portions of the growth curves overlap (Table 4.3). For *N. fowleri*, increasing the temperature from 20° to 30°C caused an increase in the growth rate of 3-fold (DAMIANO) to 7- to 10-fold (SWL 14715, NORTHCOTT). For the same temperature increase, growth rate of *N. gruberi* increased only 1.7-fold. Thus while 30°C is near optimal for the strain of *N. gruberi* studied here, the relative change in growth rate of *N. fowleri* as temperature increased to 30°C was higher.

The estimates of generation times at optimal temperature (Table 4.3 - derived from interpolated k_{24} values) do not necessarily represent growth rates which occur in the field. From Figure 4.6 it is clear that generation time at optimal temperature will vary with the same conditions that determine the slope 'a' of the linear portion of the growth curve described in Equation 4.1.

Since the upper limit of temperature tolerance of *N. gruberi* isolates varied by up to 5°C in either direction from the reference

strain (see Section 4.2), further experiments are needed to determine the range of values of T_L and Q_{10} for this species. Other experiments could indicate whether T_L and Q_{10} values calculated for the DAMIANO isolate are unusually low for *N. fowleri;* the estimated gap between optimal temperature and upper limit of tolerance was greater than for any other isolate. While *N. lovaniensis* has a similar upper limit of temperature tolerance to that of *N. fowleri,* there is no published information concerning its lower temperature limit. T_L and Q_{10} values for this species will be important in determining the relative abundance of *N. fowleri* and *N. lovaniensis* under similar conditions.

From the growth curves illustrated, an explanation of the seasonal changes in abundance of *Naegleria* species can be formulated in terms of T_L and Q_{10} values. Once water temperatures fall below approximately 18°C in autumn, *N. fowleri* trophozoites die. A proportion of *N. fowleri* cysts, more resistant to low temperature, survive several months of temperatures unfavourable for growth, to excyst as water temperatures rise in spring or early summer. There is potential for growth of *N. gruberi* throughout the year. However, as water temperature continues to rise in summer, the growth rate of (excysted) *N. fowleri* increases more rapidly than the growth rate of *N. gruberi*.

This explanation is obviously a simplification. Temperature will influence *Naegleria* species in ways other than those described by the growth curves.

Temperature coefficients and absolute rates for death of trophozoites and cysts at low temperatures will be important in determining the base from which *N. fowleri* numbers increase in summer. Whether or not a significant population of *N. fowleri* can persist in a particular water body may depend on the length of the period during which water temperature remains below 18°C.

Conditions in a natural water body will not always favour log-phase growth of *Naegleria* species, and other temperature-dependent processes will influence their numbers. Temperature coefficients of encystment and excystment will be particularly important. For example, the influence of rising temperatures in early summer would not be the same if the temperature threshhold for excystment of *N. fowleri* were higher than the lower temperature limit for growth.

It is possible that, at least in an undisturbed water body, *Naegleria* species enter the water column solely or largely as flagellates. Das (1974) explained the relative incidence of infections by *N. fowleri* and *Acanthamoeba* species in terms of the activity of *Naegleria* flagellates. The high frequency of isolation of *Naegleria* species, compared with *Acanthamoeba* species, from all reservoirs sampled in this study (see Figures 5.14, 5.17 etc.) could be explained by this argument. Certainly *Naegleria* trophozoites appear less bouyant than *Acanthamoeba* trophozoites on microscopic examination, and *Acanthamoeba* species were more common than *Naegleria* species in flowing water in the River Murray (Figures 5.12, 5.13). Weik and John (1976) described growth of *N. fowleri* in agitated cultures using a nutrient medium, but it seems likely that phagocytosis, if not division itself, is a substrate-dependent activity.

If this argument is correct, then relative abundance of *N. fowleri* and other *Naegleria* species in a freshwater body will depend strongly on the temperature coefficients of the amoeba-flagellate transformation and on the influence of temperature on the duration of the flagellate stage.

Before seasonal behaviour of populations of *Naegleria* species can be explained in detail, mathematical description of the influence of temperature on a number of physiological processes will be necessary. A partial explanation of seasonal changes in abundance has been provided here, in terms of the lower limits of temperature tolerance

and temperature coefficients of growth. A major objective of any future field study should be estimation of absolute numbers of cysts and trophozoites of *N. fowleri* and the nonpathogenic *Naegleria* species.

5. CONTROL OF *NAEGLERIA* AND *ACANTHAMOEBA* BY DISINFECTION

5.1 INTRODUCTION

The association of infections by *Naegleria* with community swimming pools (Cerva and Novak, 1968) and public water supplies (Anderson and Jamieson, 1972a) has led to concern about disinfection by authorities responsible for these community facilities. In many cases, disinfection is already practised to comply with standards for bacteriological quality of water or requirements of local health inspection. Although less closely regulated, privately owned swimming pools are usually disinfected.

Thus it is important to know the suitability of disinfectants and of established disinfection practices for these amoebae.

Chlorination is the most widely used method of disinfection of public water supplies and swimming pools. The term 'chlorination' is generally used for disinfection by chlorine gas, by sodium or calcium hypochlorite and by monochloramine.

In Australia, chlorine gas is normally used for continuous disinfection of larger public water supplies and municipal swimming pools. Equipment used for gas chlorination of a water supply usually allows automatic control of the chlorine dose with changes in flow, and in some cases, with changes in water quality.

Sodium hypochlorite, Na OCl, which is stable only in solution, is often used for continuous chlorination of smaller water supplies, and for disinfection of contaminated storage tanks. Calcium hypochlorite, $Ca(OCl)_2$, is stable as a solid, and is easily stored and transported. Thus it is often used for disinfection of water storages in remote areas, and is commercially available for disinfection of domestic swimming pools.

Chloramination is the disinfection method used for many public water supplies in the United States of America, but is not widely used in Australia.

5.2 CHEMISTRY OF CHLORINE IN WATER

Chlorine dissolves in water forming equimolar amounts of HCl and HOCl (hypochlorous acid).

$$Cl_2 + H_2O \longrightarrow HCl + HOCl \qquad \text{(Eq. 5.1)}$$

Dissociation of HOCl, a weak acid, is pH-dependent and incomplete at pH values usually encountered in natural waters.

$$HOCl \underset{H_2O \quad H_3O^+}{\overset{^-OH \quad H_2O}{\rightleftharpoons}} {}^-OCl \qquad \text{(Eq. 5.2)}$$

(hypochlorite ion)

Hypochlorous acid and the hypochlorite ion are the active molecular species in disinfection, which is an oxidative process. For most microorganisms which have been tested, the effectiveness of chlorine is greater at lower pH values (White, 1972). There are two apparent reasons for this observation. The electrochemical potential (E_0) of HOCl is considerably higher than that of the ^-OCl ion (Chang, 1971a) ie. it is a stronger oxidizer. It is also believed that HOCl, uncharged and with a molecular bulk similar to that of H_2O, penetrates bacterial cell walls more rapidly than the ^-OCl ion.

Unless a natural water is extremely well buffered, the complete dissociation of HCl which occurs after chlorine gas dissolves causes an initial fall in pH. No appreciable pH change occurs on addition of NaOCl or $Ca(OCl)_2$ to water, and the position of the $HOCl/^-OCl$ equilibrium depends on the pH of the water being disinfected.

The concentration of chlorine-based disinfectants is expressed in terms of the concentration (w/v) of the chlorine itself. Hence a solution of $Ca(OCl)_2$ has twice the 'available chlorine' (in $mg.l^{-1}$) of an equimolar solution of NaOCl.

Since disinfection is an oxidative process, the chlorine concentration falls from the initial level, the 'chlorine dose', to an extent determined by the number and bulk of organisms present and on the concentration of NH_3 and organic compounds which are oxidized by chlorine. The concentration at any instant during disinfection is known as the 'chlorine residual' and is an important parameter in the monitoring of disinfection in practice.

The extent by which the chlorine concentration is reduced is referred to as the 'chlorine demand' of the water which is being disinfected. Chlorine demand is an empirical quantity, measured as the difference between the chlorine dose and the free chlorine residual after an arbitrary contact time, usually 30 minutes.

The term 'free chlorine' is used in reference to HOCl and ^{-}OCl, which can be titrated as the 'free chlorine residual' (see Section 2.4).

Compounds of chlorine and ammonia (chloramines) also have disinfectant activity, and are referred to as 'combined chlorine'. They are formed in the following reactions:

$$NH_3 + HOCL \rightleftharpoons NH_2Cl + H_2O \qquad \text{(Eq. 5.3)}$$

monochloramine

$$NH_3 + 2HOCl \rightleftharpoons NHCl_2 + 2H_2O \qquad \text{(Eq. 5.4)}$$

dichloramine

$$NH_3 + 3HOCl \rightleftharpoons NCl_3 + 3H_2O \qquad \text{(Eq. 5.5)}$$

nitrogen trichloride

The relative concentrations of the chloramine species depends on pH and the initial HOCl : NH_3 ratio (White, 1972). The efficiency of chloramines in disinfection has been estimated, for various micro-organisms, at 1 to 5% of that of hypochlorous acid (White, 1972). Estimation of the combined chlorine residual is important where NH_3

comprises the greater part of the chlorine demand (as in disinfection of wastewater) and where chloramination is practised.

Chloramination, achieved by successive addition of NH_3 and chlorine, is used in disinfection of many water supplies in the U.S.A. where the initial level of contamination is low and sufficient contact time is available to allow for the lower efficiency of chloramines. The advantages are that a chloramine residual is more persistent than a similar free chlorine residual, owing to their relative electrochemical potentials. A number of chemical problems associated with free residual chlorination, including corrosion, are also avoided by use of chloramines.

5.3 DISINFECTION EXPERIMENTS - LABORATORY METHODS

A study of the susceptibility of several *Naegleria fowleri* strains to chlorine was carried out in the laboratory to provide a basis for disinfection of public water supplies and swimming pools in South Australia. A smaller number of experiments using *N. gruberi* and *Acanthamoeba* strains is also reported here. The *Naegleria* and *Acanthamoeba* strains studied are specified in the captions to the figures; their origins and a reference number to the American Type Culture Collection or the Culture Centre of Algae and Protozoa (Cambridge), where appropriate, are listed in Section 2.1.

The plaque count method (see Section 2.9) was developed to assess survival of amoebae as accurately as possible. Using half-log dilutions from 10^0 to 10^{-2}, it was possible to estimate the number of viable amoebae (or cysts) over a 10^3-fold range, ie. 100% to 0.1% survival or lower.

A washed suspension of cysts or trophozoites (prepared as described in Section 2.2) was dosed with chlorine using a stock solution prepared by bubbling chlorine gas through distilled water. The dose was

determined accurately by titrating a dilution of the stock solution, using the ferrous/DPD method (Section 2.4) immediately before commencing the experiment. The free and total chlorine residuals during exposure of the organisms to disinfection were measured by the same method.

The volume of the suspension was kept low (50 or 100 ml in most experiments) to avoid the necessity of culturing large volumes containing pathogenic organisms. The amoebae were exposed to chlorine at a constant temperature by incubating in a heated water bath or on a platform shaker in a constant temperature room. The bath and shaker had orbital platforms set at 100 revolutions/minute for the course of the experiment to ensure intimate contact of the organisms with the chlorine.

The medium in which amoebae were suspended for exposure to chlorine varied between experiments. A sterile suspending medium was necessary because growth of microorganisms which were not metabolised by the amoebae (particularly gram-positive bacteria and moulds) obscured plaques and reduced the accuracy of the count. For initial experiments in which the plaque count method was established (Figures 5.1, 5.2), cysts of *Naegleria fowleri* were suspended in water from the River Murray at Morgan which had been sterilized by autoclaving. Apart from sterility, the suspending medium was intended to be similar to the environment in which amoebae would be exposed to initial chlorination of the Morgan-Whyalla Pipeline.

However, flocculation of suspended solids on autoclaving made dispensing of equivalent suspensions difficult; changes in pH may also occur on autoclaving. For the suspending medium used in further experiments, water from the River Murray at Morgan was passed through an ultraviolet water sterilizer (Oliphant), which provided adequate disinfection without altering pH or producing observable changes in the physical characteristics of the water. In a few experiments *N. fowleri* cysts were suspended in simple inorganic buffers, prepared according to

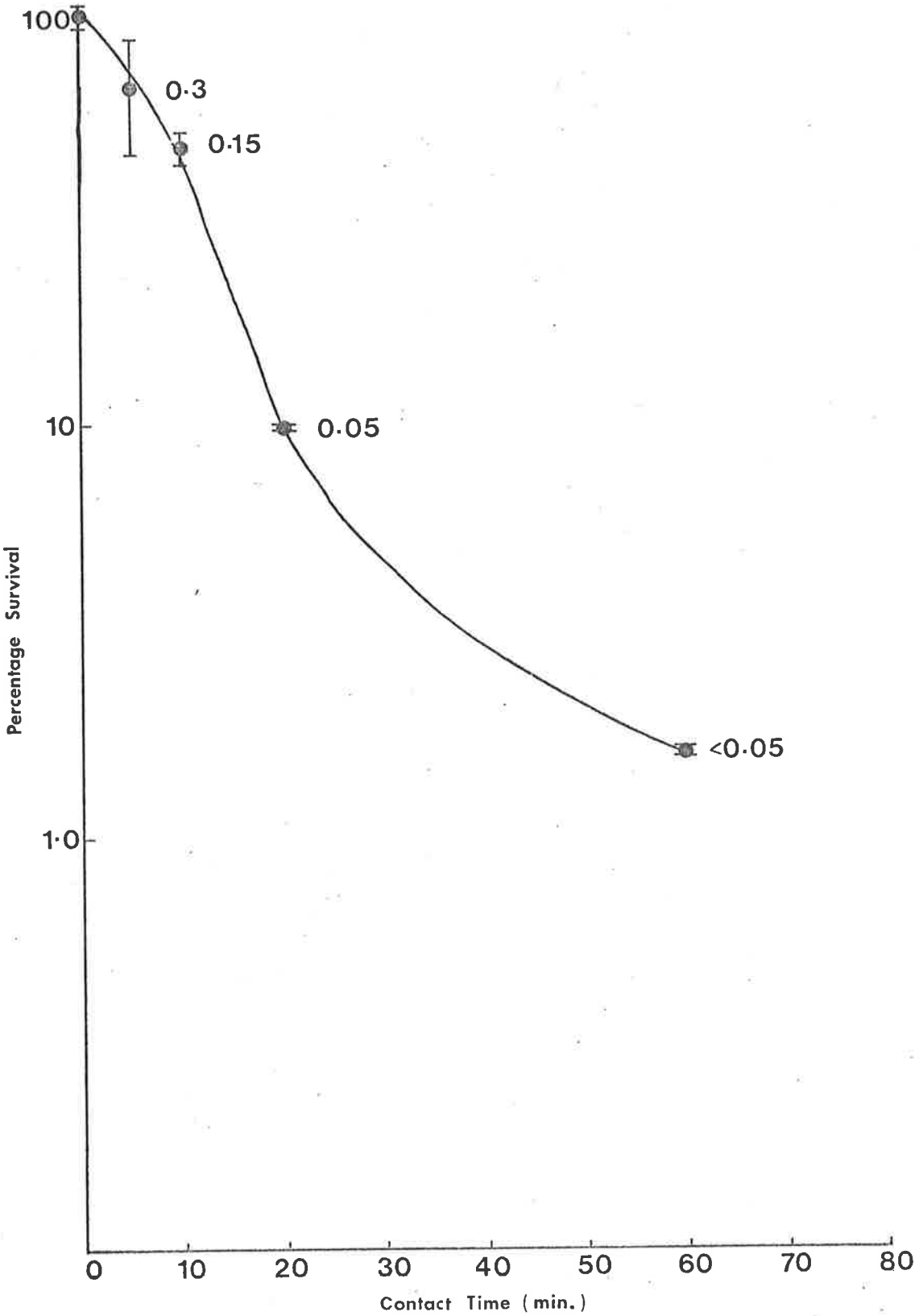


Figure 5.1 Susceptibility of *Naegleria fowleri* NHI to chlorine. 4-day cysts, chlorine dose 4.5 mg.l^{-1}. Figure beside each survival point is the free chlorine residual (mg.l^{-1}).

Survival based on mean (± s) of duplicate plaque counts.

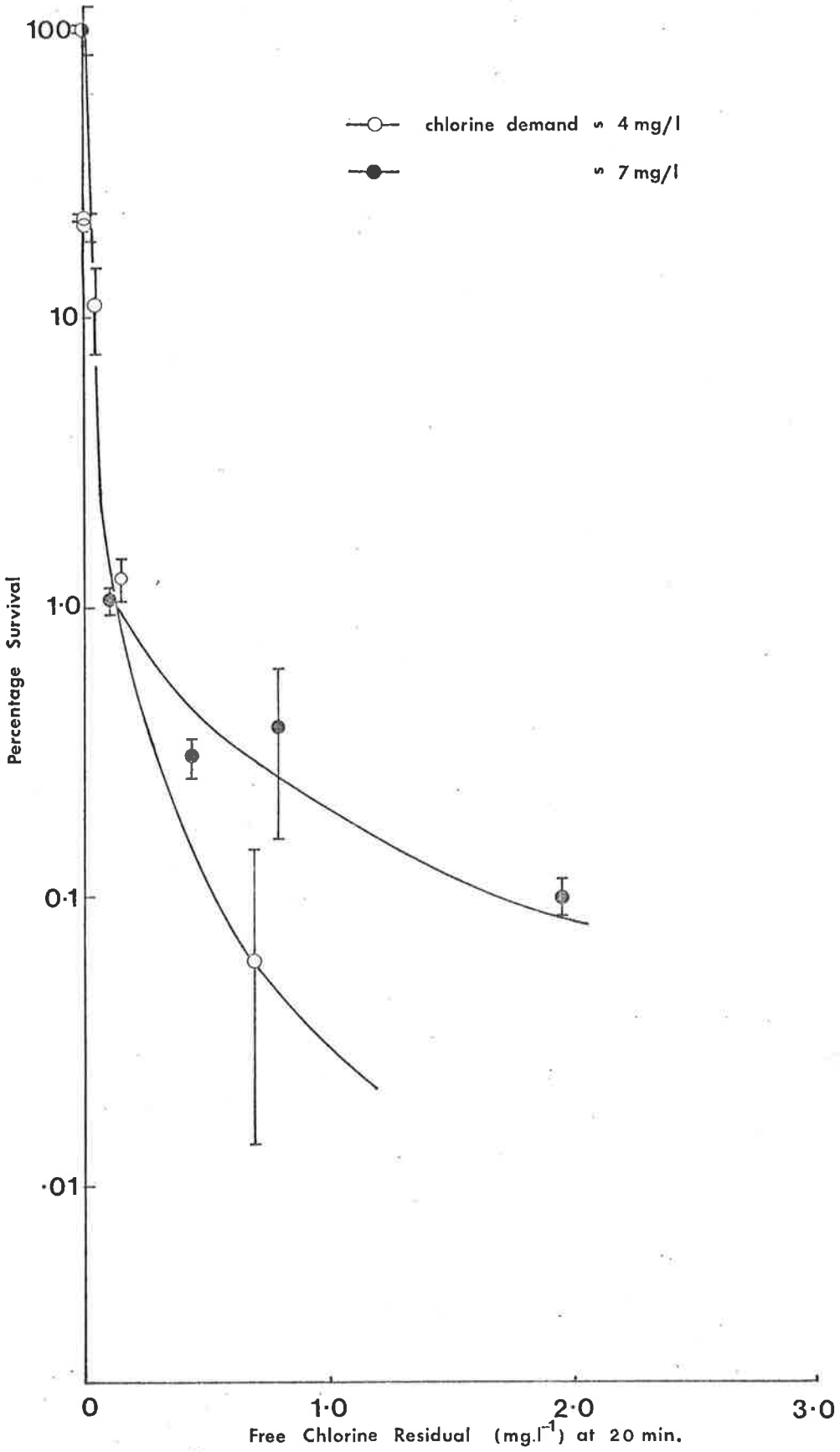


Figure 5.2 Susceptibility of *Naegleria fowleri* NHI to chlorine. 4-day cysts, varying chlorine dose.

Survival based on mean (± s) of duplicate plaque counts.

tables in Willard *et al.* (1965). It was not necessary, for example, to have the 'natural' chlorine demand for demonstration of the influence of pH and temperature on disinfection (Figures 5.4 and 5.6).

In the figure illustrating each experiment, survival values have been calculated from the mean (± standard deviation) of duplicate plaque counts, and plotted on a logarithmic scale against contact time or the free chlorine residual. In the first experiment investigating the time course of survival (Figure 5.1), a suspension of cysts was incubated under the same conditions without the chlorine dose as a 'settling control'. The number of viable cysts recovered did not change significantly over 60 minutes. In further experiments, survival was calculated as a percentage of the number of organisms present at zero time (before introduction of chlorine).

Straight lines have not been fitted to figures for survival against contact time, for reasons which are explained in a discussion of the kinetics of disinfection (Section 5.5).

5.4 EFFECT OF CHLORINE ON *NAEGLERIA*

The survival of cysts of *N. fowleri* NHI at intervals following a chlorine dose of 4.5 $mg.l^{-1}$ is illustrated in Figure 5.1. The change in concentration of chlorine (free chlorine residual) during the contact time is shown by the figure beside each survival point. Note that the free chlorine residual had fallen to the limit of sensitivity of the titration within 20 minutes, but that the number of viable cysts continued to fall.

In Figure 5.2, the survival of cysts of *N. fowleri* NHI at 20 minutes is plotted against the free chlorine residual, after exposure to a range of chlorine doses. Two experiments, in which the chlorine demand differed, are illustrated (cf. comment on suspending media in Section 5.3). The difference in slope of the survival curves probably

reflects the difference in chlorine demand, but in both experiments the number of viable cysts was reduced to 1% by a dose which provided a free chlorine residual of approximately 0.2 $mg.l^{-1}$ at 20 minutes.

The susceptibility of a pathogenic *Naegleria* strain isolated in South Australia, *N. fowleri* MORGAN, to a range of chlorine doses is illustrated in Figure 5.3. At free chlorine residuals of 1.4 and 2.2 $mg.l^{-1}$, plaques were absent from the undiluted (10^{0}) plates, ie. survival was less than 0.01%. However, single plaques grew at $10^{-3/2}$ and 10^{-1} and presumably represent the chance distribution of single cysts during dilution. Points representing the conservative interpretation of these single plaques have been included, without confidence limits, in Figure 5.3. In this experiment, 99% of cysts were killed by a chlorine dose sufficient to provide approximately 0.4 $mg.l^{-1}$ free chlorine residual at 30 minutes.

The influence of pH on susceptibility of *N. fowleri* MORGAN to chlorine is illustrated in Figure 5.4. Suspensions of equal numbers of cysts and the same chlorine demand were buffered at pH 7.0 and pH 8.5 using phosphate buffer. A direct comparison of survival was made using the same chlorine dose (2.8 $mg.l^{-1}$), and the changes in free and total chlorine and pH are shown in Figure 5.5.

The survival of *N. fowleri* MORGAN exposed to chlorine is influenced by pH in a manner predictable from the chemistry of chlorine in water, assuming that hypochlorous acid is the most important 'disinfecting species' (see Section 5.2). *N. fowleri* MORGAN was significantly more susceptible to chlorine at pH 7.0 than at pH 8.5, corresponding with a more rapid fall in the free chlorine residual.

The importance of pH in disinfection in the field depends on the contact time available between the organisms and the chlorine. The greater stability of the free chlorine residual at alkaline pH (Figure 5.5) would provide a similar degree of disinfection after a

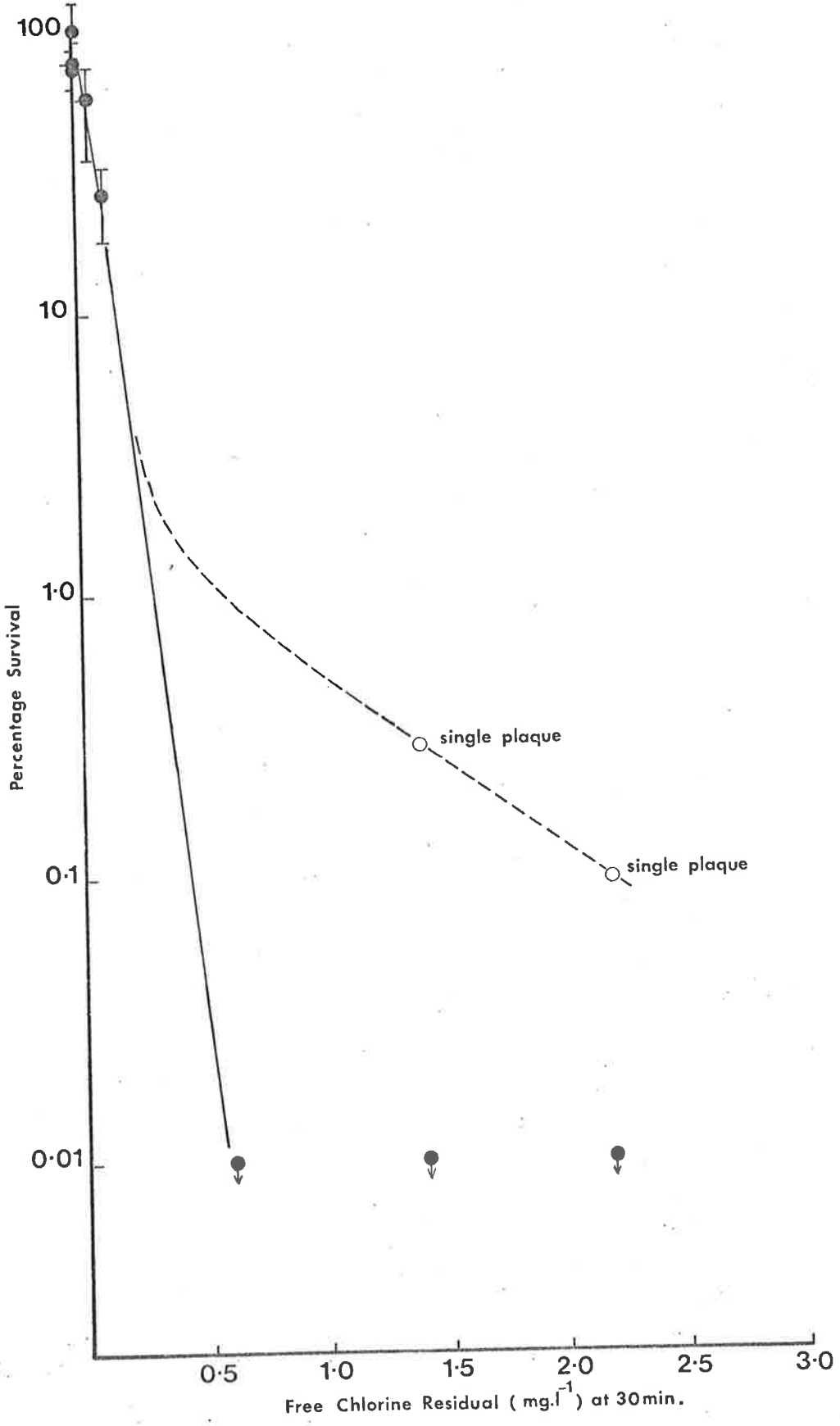


Figure 5.3 Susceptibility of *Naegleria fowleri* MORGAN to chlorine. 4-day cysts, varying chlorine dose. ↓ below limit of detection Survival based on mean (± s) of duplicate plaque counts.

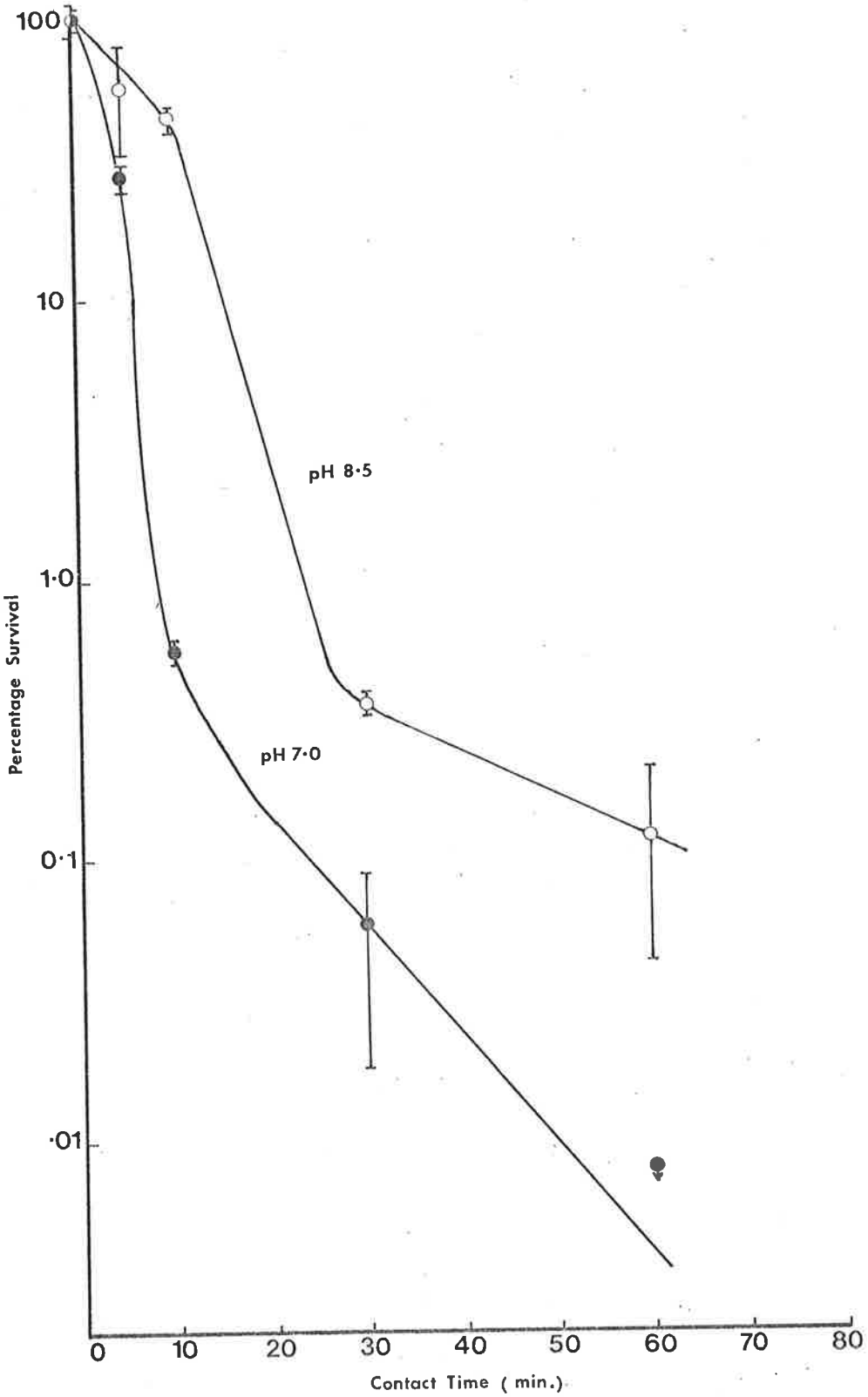


Figure 5.4 Influence of pH on susceptibility of *Naegleria fowleri* cysts to chlorine.

4-day cysts, MORGAN strain. Chlorine dose 2·8 mg.l^{-1}

Survival based on mean (± s) of duplicate plaque counts.

● below limit of detection

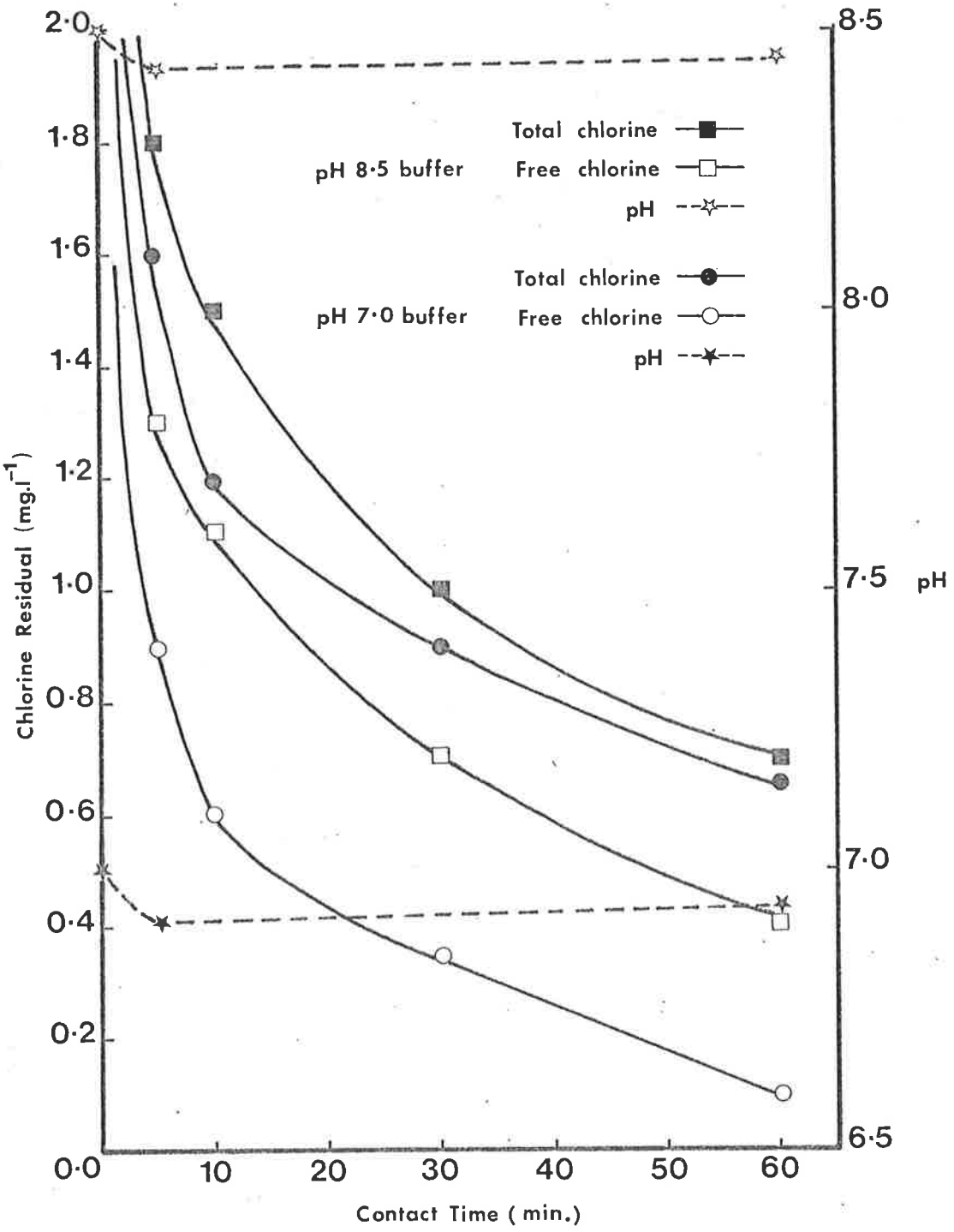


Figure 5.5 Chlorine residuals and pH after chlorination. 4-day cysts, MORGAN strain. Chlorine dose 2·8 mg.l^{-1}

longer exposure time.

The survival of cysts of *N. fowleri* MORGAN after exposure to chlorine at 30°C and 17.5°C is illustrated in Figure 5.6. Water temperatures in reticulated water supplies in Port Augusta, Port Pirie and Kadina are below 20°C for much of the year, but reach 30°C on occasions during most summers. Chlorine killed cysts of *N. fowleri* more rapidly at 30°C, a temperature which is likely to occur during the season when amoebic meningitis is a risk in South Australia.

Although disinfection was less rapid at 17.5°C, higher free chlorine residuals persisted (Figure 5.7) and provided contact time is adequate, a similar degree of disinfection could be expected. Domestic demand for water is lower during cooler months, for example, and reduced flow in the water supply usually allows longer 'detention times' between disinfection and delivery.

The applicability of these experiments depends on how representative the cysts, prepared in the laboratory on solid media, were of cysts occurring in water under natural conditions. An attempt was made to assess the influence of growing *N. fowleri* on solid media on the susceptibility of their cysts to chlorine, by harvesting cysts 6 days and 20 days after respreading with *E. coli*. '20-day' cysts were somewhat more susceptible to chlorine than '6-day' cysts (Figure 5.8).

It was impractical to prepare cysts of *Naegleria* in liquid culture. *Naegleria* do not encyst readily in Fulton's Medium A (Fulton, 1970), and no equivalent to the 'encystment medium' used for *Acanthamoeba castellanii* (Neff *et al.*, 1964) has been developed. In most of the experiments described, cysts harvested 4 days after respreading were used.

Two European studies of the effect of chlorine on *Naegleria* and *Acanthamoeba* have been published (Derreumaux *et al.*, 1974; de Jonckheere and van de Voorde, 1976). In both studies, survival was assessed by filtering a volume of the suspension and inverting the membrane filter

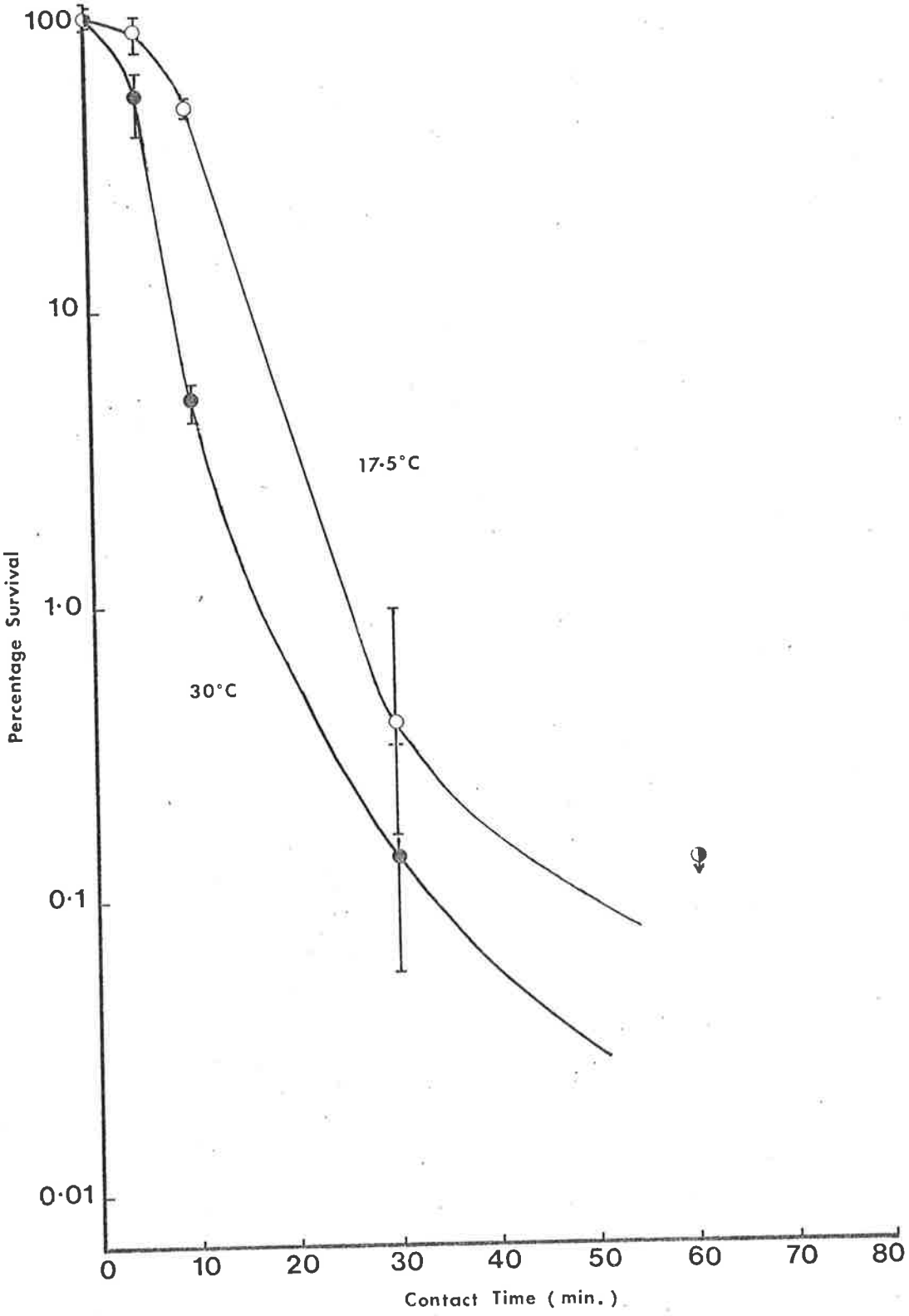


Figure5.6 Influence of temperature on susceptibility of *Naegleria fowleri* cysts to chlorine.

4-day cysts, MORGAN Strain. Chlorine dose 2·4 mg.l^{-1}

Survival based on mean (± s) of duplicate plaque counts.

● below limit of detection

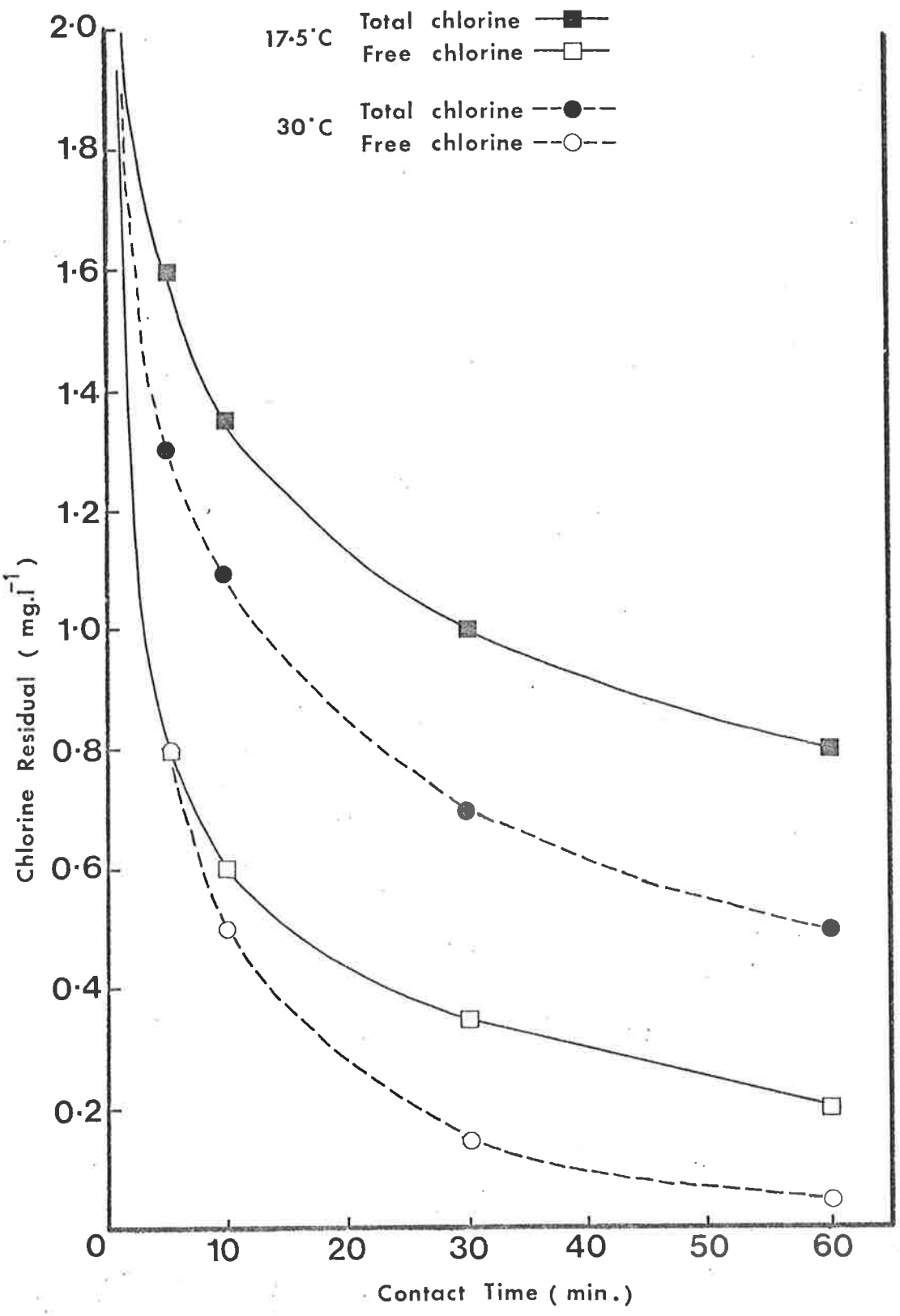


Figure 5.7 Chlorine residuals after chlorination.

4-day cysts , MORGAN strain. Chlorine dose 2·4 mg.l^{-1}

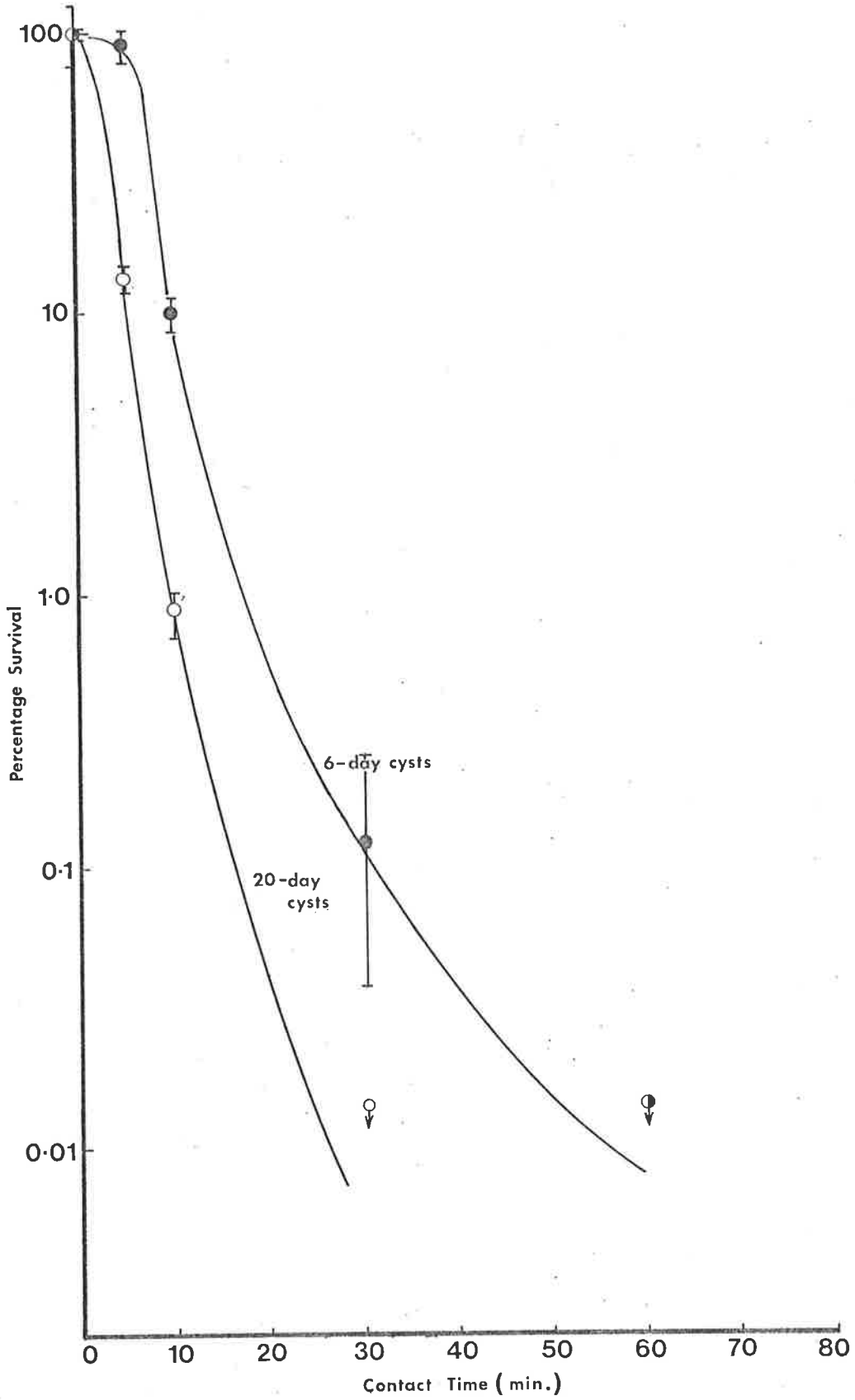


Figure 5.8 Influence of age on susceptibility of *Naegleria fowleri* cysts to chlorine. MORGAN Strain; chlorine dose 2·3 $mg.l^{-1}$ Survival based on mean (± s) of duplicate plaque counts. ⏷ below limit of detection

on agar spread with bacteria. Cultures were examined daily over a suitable incubation period and recorded as 'positive' or 'negative'.

On the basis of the inoculum and sample size used by de Jonckheere and van de Voorde (1976), 'negative' cultures would have occurred when survival fell below 1 in 10^4 (assuming 100% recovery from the membrane filters). Derreumaux *et al.* (1974) using varying numbers of cysts in their inocula, and negative cultures would have occurred at survivals below 1 in 500 to 1 in 5×10^3.

The authors of both papers expressed the effectiveness of chlorine in terms of the contact time required for survival to fall below the level detected by their recovery method. Thus their results are not easily compared with survivals estimated from the plaque counts in these experiments.

Plaque counts were used by Chang (1978) in experiments on the effect of chlorine and iodine on *Naegleria fowleri*. These experiments were designed to provide information on the kinetics of disinfection, and again the results are expressed as the time required for a given degree of disinfection. For a public water supply where there are constraints on the contact time available for disinfection and the chlorine demand may vary, it is more practical to think in terms of varying the chlorine dose to achieve adequate disinfection.

Although the effectiveness of chlorine varies somewhat with the chlorine demand of the water, under most conditions the survival of cysts of pathogenic *Naegleria* can be reduced to less than 1% by a chlorine dose sufficient to provide a free chlorine residual of 0.4-0.5 $mg.l^{-1}$ after 30 minutes.

5.5 KINETICS OF DISINFECTION

Disinfection experiments with some microorganisms suggest that interaction with the disinfectant is similar to a chemical reaction with

pseudo - first order kinetics (Chang, 1966). At a given concentration of disinfectant, survival is an inverse exponential function of time. An assumption of this model, that the concentration of the disinfectant remains constant throughout the contact time, was not fulfilled in experiments presented here (e.g. Figures 5.5, 5.7). For an oxidative disinfectant such as chlorine to satisfy this assumption, the demand for the disinfectant must approach zero. This would require idealized chemical conditions and a very low density of microorganisms, reducing the sensitivity of the test. An alternative was used by Farooq *et al.* (1977) to study the kinetics of disinfection by ozone. Experiments were performed in special apparatus with continuous and controlled introduction of ozone to maintain its concentration.

Such a refinement did not seem warranted in this study. The main objective was to examine the chemical and physical variables which influence the effectiveness of chlorine against *Naegleria* and *Acanthamoeba* and to determine effective doses under conditions which apply in South Australia.

Analysing survival curves drawn from several studies of viruses, Chang (1966) explained any departure from this model in terms of 'clumping' of viruses. An aggregate of viruses could appear as a single plaque until every individual virus in the aggregate had been killed. The shape of the curve would be determined by the relative number and size distribution of aggregates.

In experiments presented here, $\log_{10}$ percentage survival appeared not to be a linear function of time (eg. Figures 5.4, 5.6). However, examination of cysts using a haemocytometer immediately before exposure to disinfection (see Section 5.3) showed that mixing was adequate and there was no appreciable aggregation.

In several experiments, survival at the shortest contact times (5, 10 minutes) suggested a convex survival curve which might be interpreted

as a 'lag' in the disinfection process. An experiment was carried out to elucidate the initial kinetics of the action of chlorine on *Naegleria* cysts. Four-day cysts of *N. fowleri* and *N. gruberi* were exposed to 2.0 $mg.l^{-1}$ free chlorine at pH 7.0. Samples were taken and the action of chlorine halted by sodium thiosulphate ($Na_2S_2O_3$) at accurately measured time intervals during the first ten minutes of contact.

The number of viable cysts of *N. fowleri* fell at a rate which increased during the first ten minutes of contact with chlorine (Figure 5.9). Although Chang (1978) interpreted the effect of chlorine on *N. fowleri* in terms of a pseudo-first order model, it appears from this experiment that survival is not initially an inverse exponential function of time. If the lag in disinfection represents time required for chlorine to penetrate the cysts, the difference between this survival curve and those observed for bacteria and viruses (often fitting the pseudo-first order model closely) could be explained in terms of their vastly different cell volumes and perhaps the composition of the cell (or cyst) wall.

For *Naegleria gruberi* exposed to the same concentration of chlorine, there was little change in the number of viable cysts until after five minutes of contact with chlorine (Figure 5.9). This represents a lag in disinfection more marked than that observed for *N. fowleri*, and confirms the observations of de Jonckheere and van de Voorde (1976) that *N. gruberi* is less susceptible to chlorine than *N. fowleri*. Since *N. gruberi* is also less susceptible to desiccation than *N. fowleri*, an explanation of the lag in terms of the permeability of the cyst wall to small molecules (in this case, HOCl) would be credible.

In most of the disinfection experiments with *N. fowleri*, a 'tailing-off', representing a slower rate of disinfection, occurred at longer contact times. This is consistent with the falling concentration of chlorine during the course of the experiment (compare Figures 5.4, 5.5).

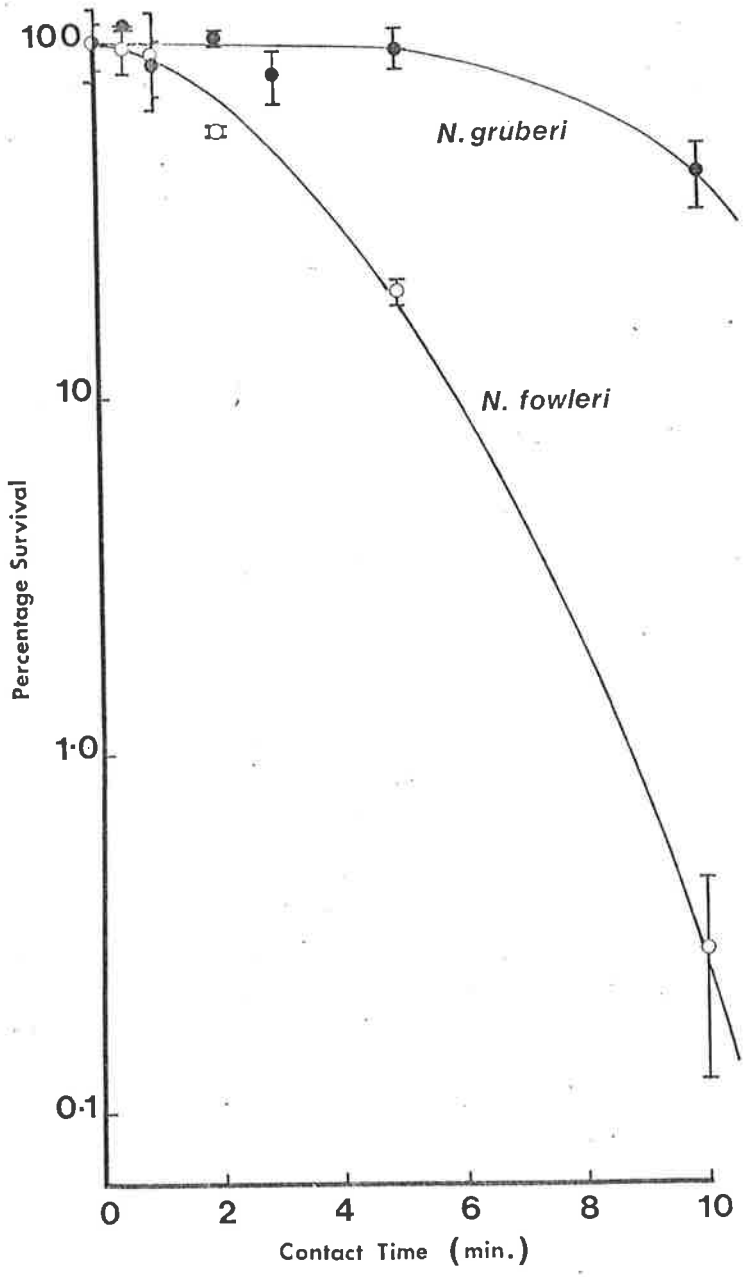


Figure 5.9 Susceptibility of *N. gruberi* and *N. fowleri* cysts to chlorine : initial kinetics. Strains SWL 10306/37, NORTHCOTT. Chlorine dose 2 mg.l^{-1} Survival based on mean (± s) of duplicate plaque counts.

5.6 EFFECT OF CHLORINE ON *ACANTHAMOEBA*

Disinfection experiments were carried out using two morphologically distinct strains of *Acanthamoeba*.

The susceptibility of cysts and trophozoites of *Acanthamoeba castellanii* NEFF to chlorine is illustrated in Figure 5.10. The number of viable cysts fell slowly, and at 60 minutes (free chlorine residual 2.6 $mg.l^{-1}$) survival was 37.5%. The suspension of trophozoites, prepared from plate cultures, contained a small number of cysts (estimated at below 1.0% in the preliminary haemocytometer count). The number of viable 'trophozoites' fell rapidly to approximately 0.1% where the slope of the survival curve changed. Viable organisms detected between 10 and 30 minutes were interpreted as representing the small proportion of cysts seen in the preparation before the experiment commenced.

In another experiment, the susceptibility to chlorine of *Acanthamoeba polyphaga* FF-1 (a strain isolated from a mammalian cell culture - see Section 2.1) was compared with that of *Naegleria fowleri* at pH 7.0. Exposed to a chlorine dose of 4.0 $mg.l^{-1}$ at a very low chlorine demand, the survival of *N. fowleri* cysts fell rapidly to below 1.0% (Figure 5.11). By contrast, approximately 60% of *A. polyphaga* cysts survived two hours contact with the chlorine.

Plate cultures of *Acanthamoeba* species tended to encyst in aggregates more than did cultures of *Naegleria* species. Since the percentage survival in all experiments is based on the number of 'plaque-forming units', interpreted as equivalent to the number of individual viable organisms, the presence of clumps of cysts may have influenced the kinetics of the survival curve (see Section 5.5). However, the proportion of cysts in clumps was small (judged on microscopic inspection) and both *Acanthamoeba* strains were clearly very resistant to chlorine.

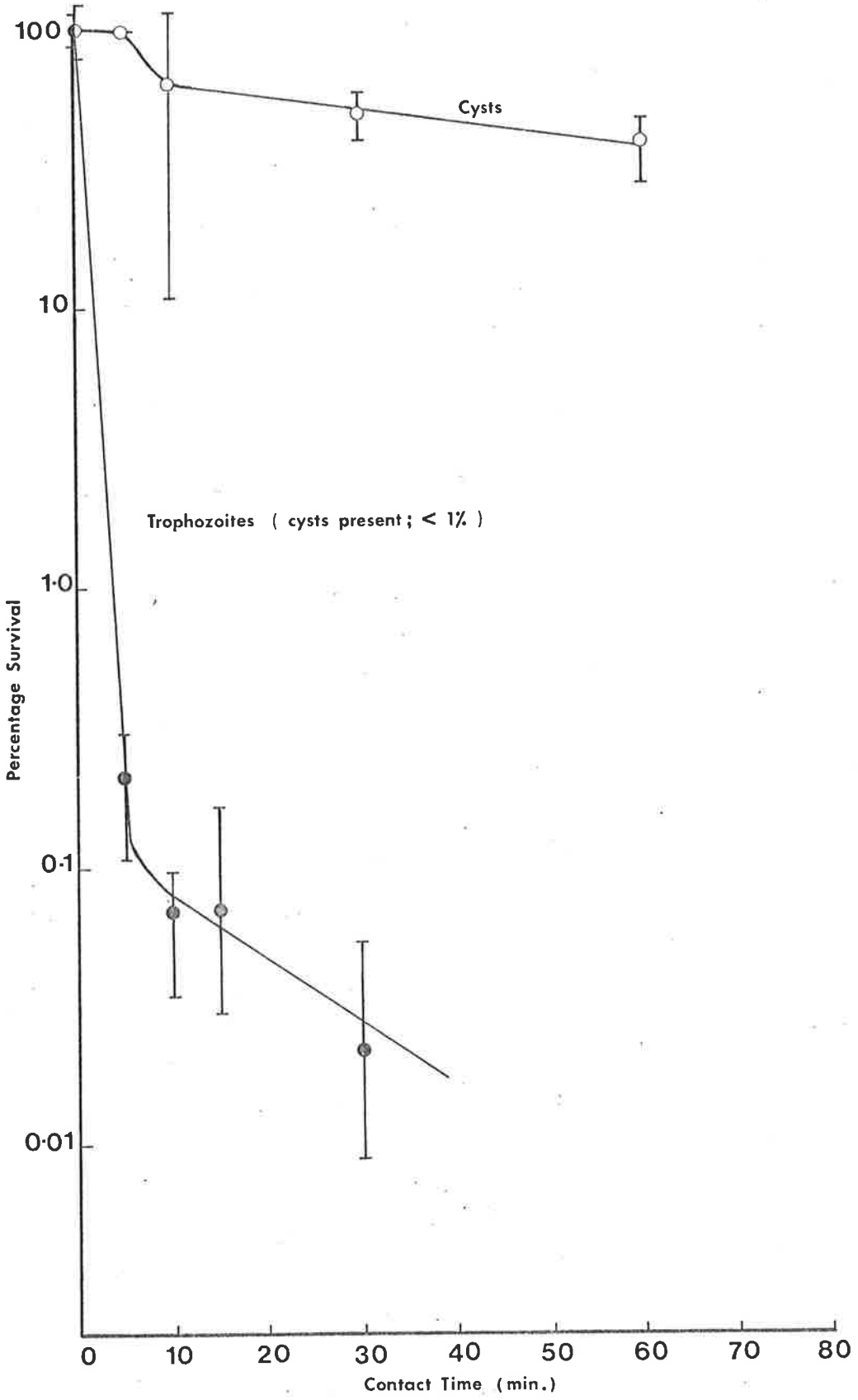


Figure 5.10 Susceptibility of ***Acanthamoeba castellanii*** trophozoites and cysts to chlorine. NEFF strain. Chlorine dose 4 mg.l^{-1}

Survival based on mean (± s) of duplicate plaque counts.

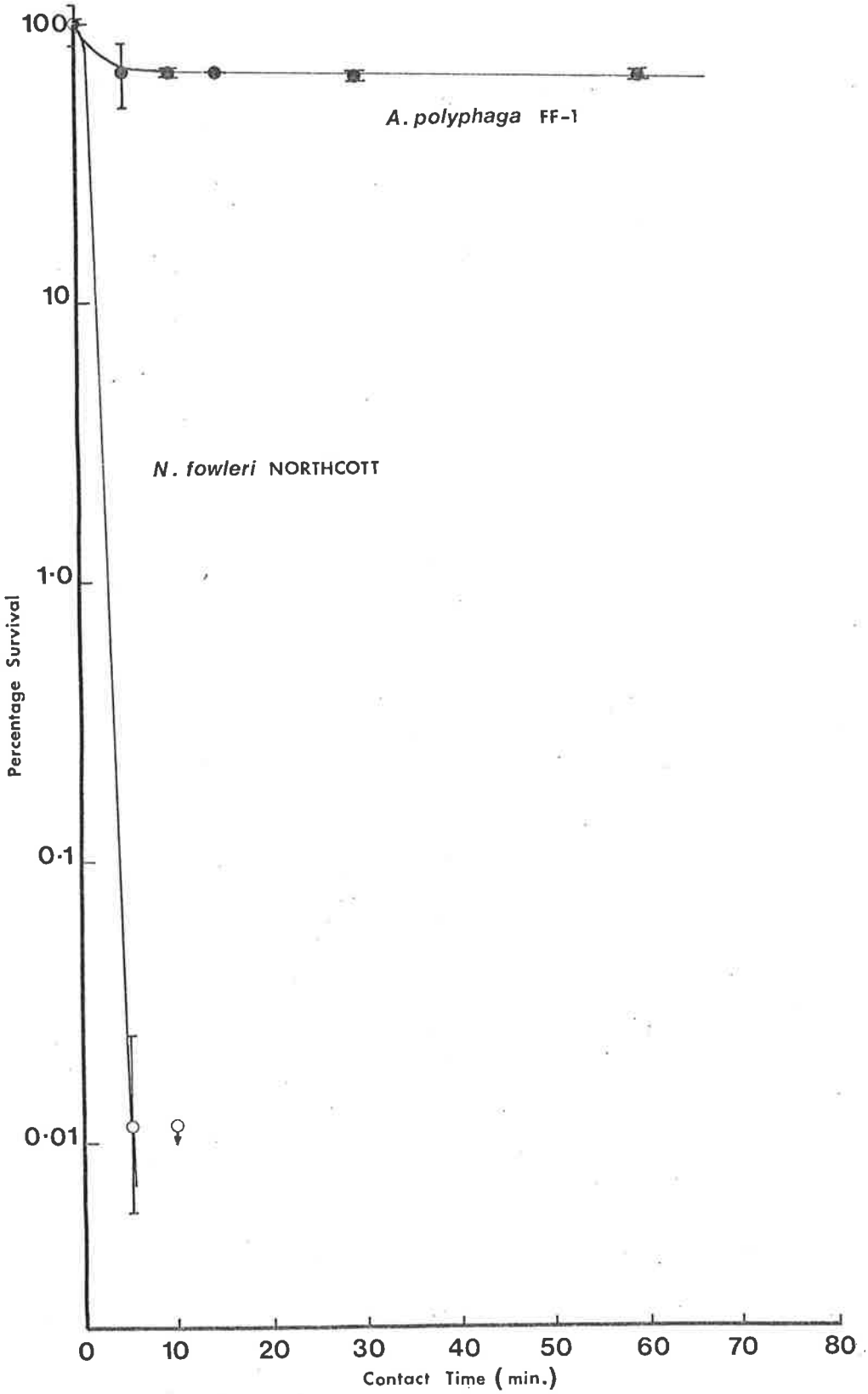


Figure 5.11 Susceptibility of cysts of *Acanthamoeba polyphaga* and *Naegleria fowleri* to chlorine. Chlorine dose 4 mg.l^{-1}

Survival based on mean (± s) of duplicate plaque counts. ♀ below limit of detection

It is interesting to note that resistance to chlorine is characteristic only of the cysts of *Acanthamoeba* species.

Acanthamoeba cysts are also far more resistant to desiccation than *Naegleria* species (Page, 1967b), suggesting again that permeability of the cyst wall to small molecules is important in determining resistance to chlorine.

5.7 EFFECTIVENESS OF CHLORINATION IN PRACTICE

A weekly sampling programme has been carried out since the 1972/73 summer, encompassing water sources and water supplies to areas of South Australia where cases of amoebic meningoencephalitis have occurred (Figure 1.1). While the primary purpose of sampling has been the week to week monitoring of water supplies and regulation of the chlorinating stations, examination of the results of sampling illustrates the effectiveness of chlorine in the field.

To provide material for comparison samples were also collected before and after disinfection at Tailem Bend, farther south on the River Murray than Morgan. Samples were also collected from three reservoirs supplying water to metropolitan Adelaide. For each sample collected, field measurements of water temperature and free and total chlorine residual were made.

Because individual samples indicate presence or absence of amoebae in 250 ml of water, the results are presented as a frequency distribution of the isolation of amoebae. The frequency of isolation of amoebae from a sample point after chlorination is presented as a histogram, with the bars representing intervals of concentration of chlorine (free chlorine residual): < 0.1 mg.l^{-1}, 0.1 - 0.45, 0.5 - 0.95, 1.0 - 2.0 and $\geqslant 2.0$ mg.l^{-1}. The height of each bar represents the percentage of samples in that range of chlorine residual. The bars are marked to indicate the percentage of samples from which amoebae

were isolated, and isolations of *Naegleria* and *Acanthamoeba* are specifically identified. Note that isolation of *Naegleria* does not exclude isolation of *Acanthamoeba* or other amoebae from the same sample.

When comparing the field and laboratory results, the reader should remember that the free chlorine residual is an instantaneous measurement of the chlorine concentration in the water, and does not specify a contact time or the absolute concentration to which the organisms have been exposed. At a particular sample point, the contact time depends on the water flow, which may vary seasonally with the demand for water.

Figure 5.12 shows the frequency of isolation of amoebae from the River Murray at Morgan and immediately after chlorination in the Morgan-Whyalla pipeline, for samples collected between 1.1.74 and 30.6.79. *Naegleria* was isolated from 41% of samples collected from the river, and was not present in any sample after disinfection. However, *Acanthamoeba* (isolated from 70% of samples of river water) was isolated from a high proportion of samples after chlorination, and was the most common amoeba isolated at high free chlorine residuals.

Naegleria was present in 38% of samples collected from the River Murray at Tailem Bend during the same period and was effectively removed by chlorination (Figure 5.13). However, *Acanthamoeba* (54% from river samples) was present in a high proportion of samples collected immediately after chlorination. The River Murray at Tailem Bend is the source of water supplied through a long pipeline to Keith, a more southerly town where no cases of amoebic meningoencephalitis have occurred.

The immediate sources of water for Port Pirie are Nelshaby Reservoir and the Morgan-Whyalla pipeline at Napperby. Water chlorinated at each of these sources mixes in a trunk main which is sampled at George's Corner. The frequency of isolation of amoebae from

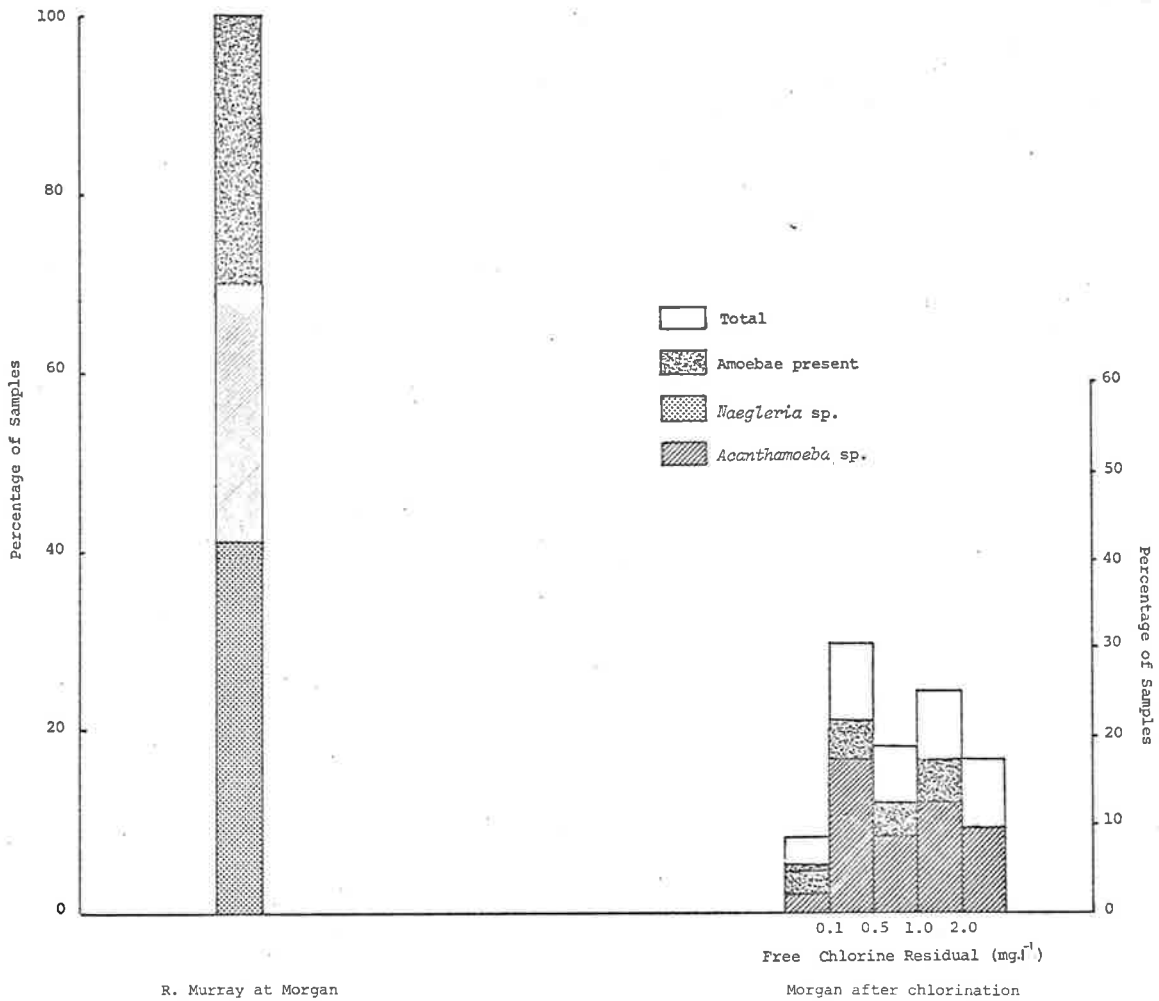


FIGURE 5.12 FREQUENCY OF ISOLATION OF AMOEBAE FROM THE RIVER MURRAY AND AFTER CHLORINATION AT MORGAN, 1.1.74 - 30.6.79

Height of each portion of the bar above the base-line represents the percentage of samples in that class.

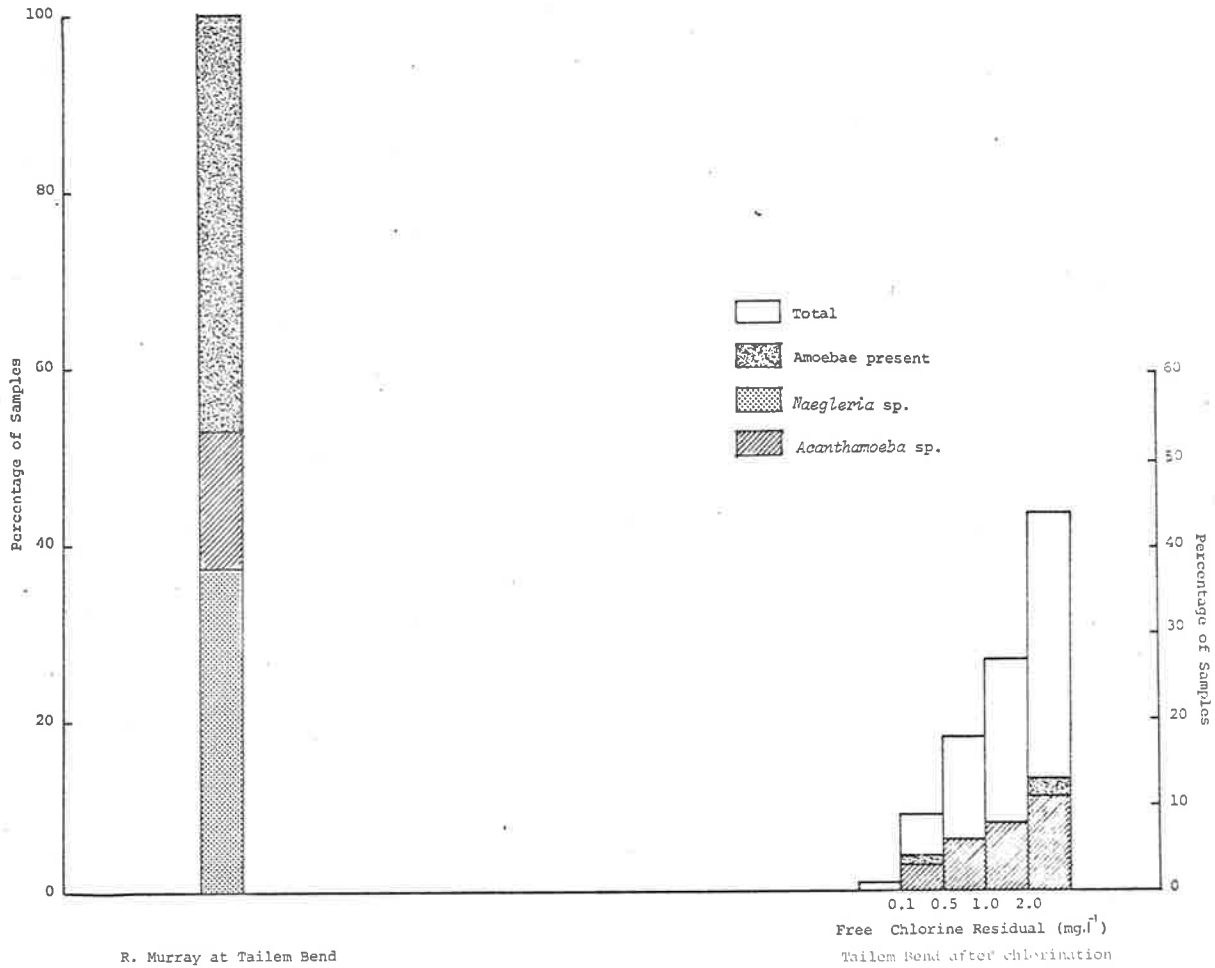


FIGURE 5.13 FREQUENCY OF ISOLATION OF AMOEBAE FROM THE RIVER MURRAY AND AFTER CHLORINATION AT TAILEM BEND, 1.1.74 - 30.6.79

Height of each portion of the bar above the base-line represents the percentage of samples in that class.

each of these locations between 1.1.74 and 30.6.79 is shown in Figure 5.14. *Naegleria* was isolated from 54% of samples collected from Nelshaby Reservoir, and *Acanthamoeba* from 8%.

Naegleria was rarely isolated from the Morgan-Whyalla pipeline at Napperby, although usually very little free chlorine persisted from chlorination at Robertstown, and amoebae were present in most samples. Water storage tanks at Gulnare and Hughes Gap are the only likely points of contamination between Robertstown and the sample point at Napperby. The amoebae most commonly isolated at Napperby - although not illustrated on the histogram - were *Vannella* species *(V. simplex, V. mira* and *V. platypodia); Vexillifera* species were also common. Since amoebae of both genera have an extremely buoyant 'floating form' and settle very slowly (Page, 1968), it seems likely that they are easily resuspended from sediments in a balance tank. Other amoebae may 'settle out' as water passes through the tank.

Chlorination at Nelshaby and Napperby resulted in removal of viable amoebae from all but 10% of samples collected from the trunk main entering Port Pirie at George's Corner. Again, *Acanthamoeba* was the most common isolate after chlorination.

The Morgan-Whyalla pipeline at Stirling North is usually the sole source of water for Port Augusta. As at Napperby, isolation of *Naegleria* or *Acanthamoeba* was relatively uncommon (Figure 5.15). The range of amoebae isolated was probably influenced by passage through balance tanks at Winninowie, and the amoebae most commonly isolated were *Vannella* and *Vexillifera* species. The frequency of isolation of amoebae after chlorination (sampled at Port Augusta Causeway) was low, although *Acanthamoeba* was occasionally present.

Between 1.1.74 and 30.6.79, samples were collected weekly from five locations on the water reticulation system of Port Pirie. There were some consistent differences between the free chlorine residuals

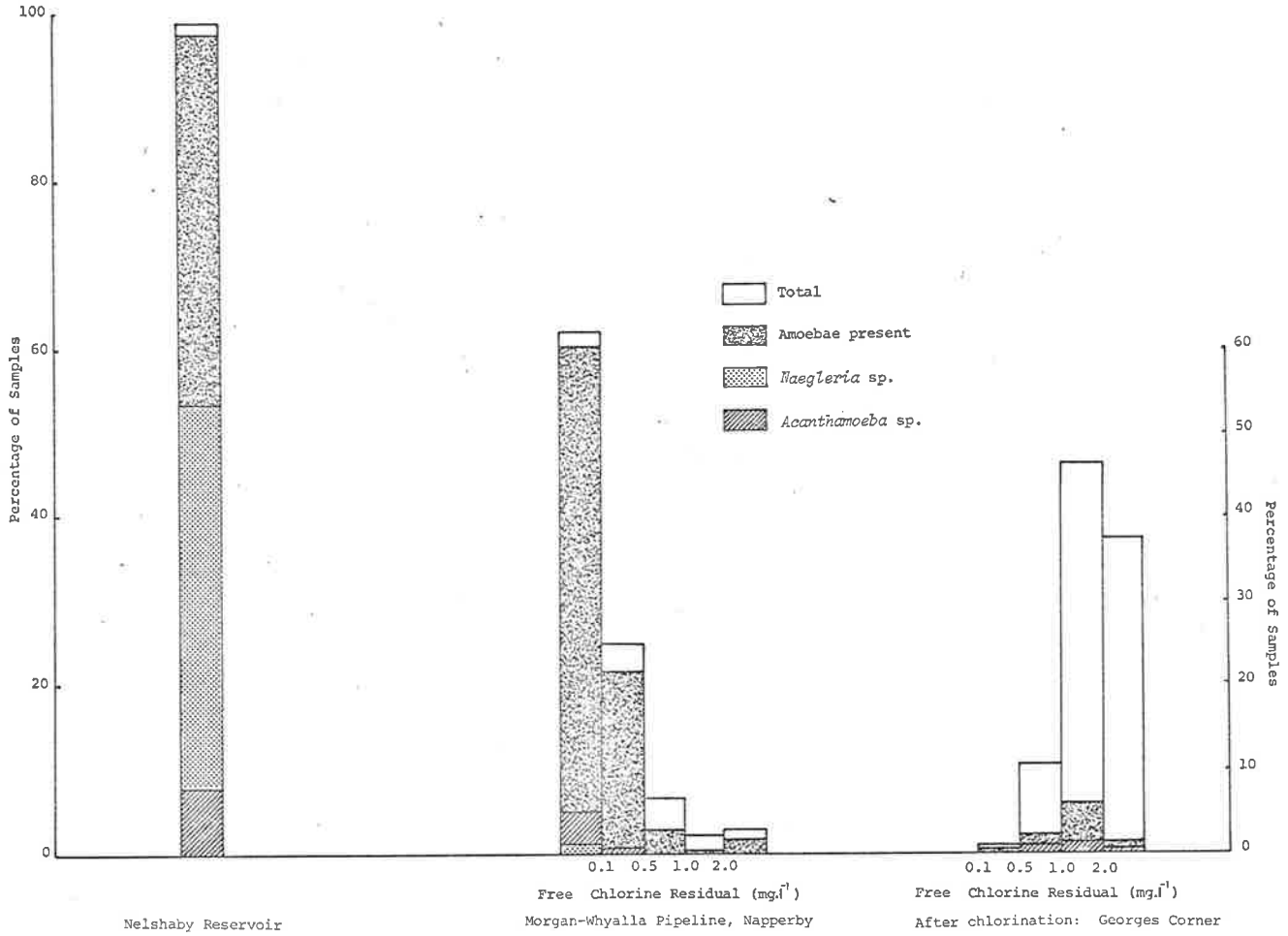


FIGURE 5.14 FREQUENCY OF ISOLATION OF AMOEBAE FROM WATER SUPPLIED TO PT PIRIE: NELSHABY RESERVOIR, MORGAN-WHYALLA PIPELINE AT NAPPERBY, AND THE TRUNK MAIN AFTER RECHLORINATION 1.1.74 - 30.6.79

Height of each portion of the bar above the base-line represents the percentage of samples in that class.

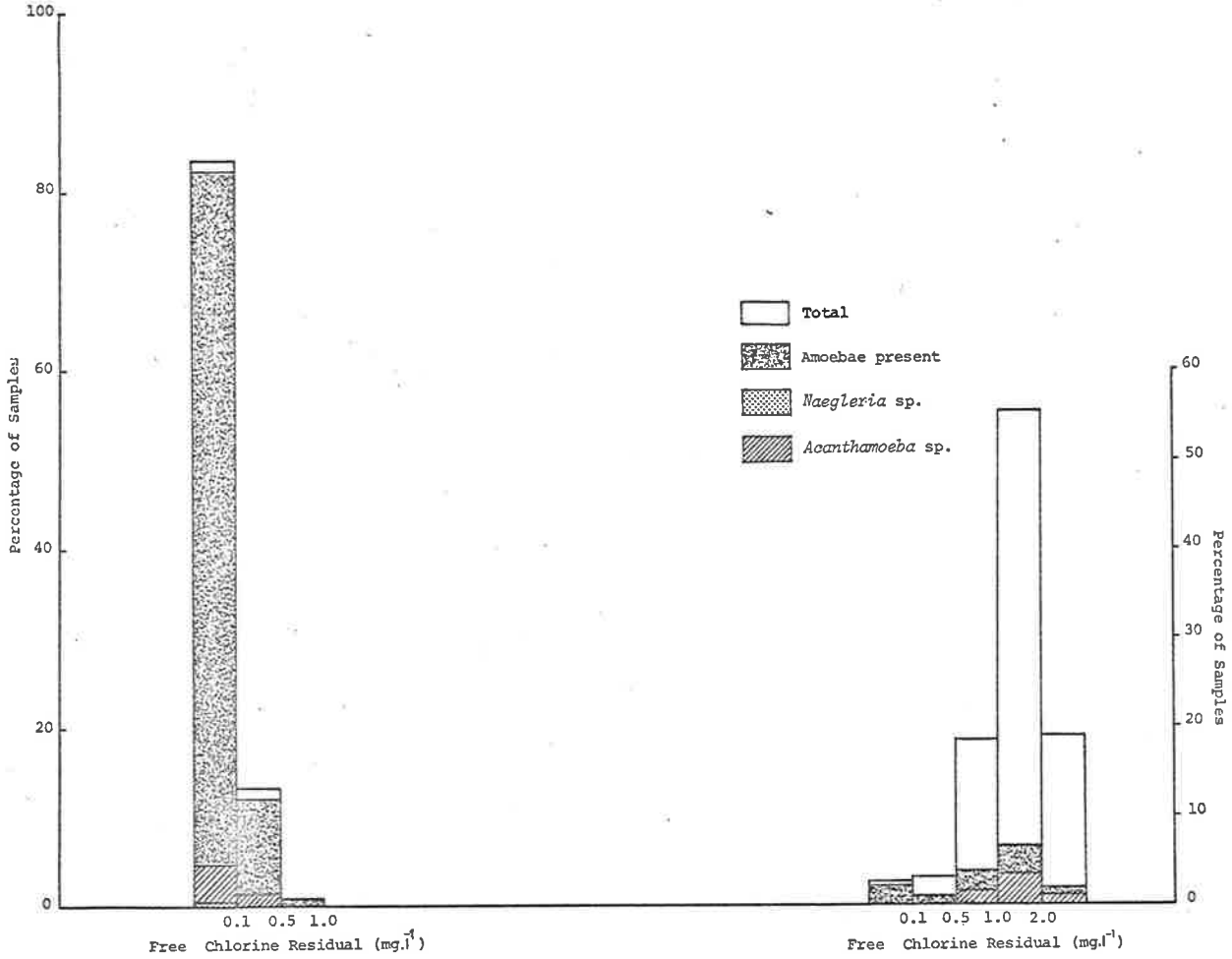


FIGURE 5.15 FREQUENCY OF ISOLATION OF AMOEBAE FROM WATER SUPPLIED TO PT AUGUSTA, BEFORE AND AFTER RECHLORINATION AT STIRLING NORTH, 1.1.74 - 30.6.79

Height of each portion of the bar above the base-line represents the percentage of samples in that class.

measured at each location which relate to the detention time from the chlorinator. Water reaching the extremities of the system and areas of lower flow generally had lower free chlorine residuals. The frequency of isolation of amoebae at different locations seemed to reflect simply the difference in level of free chlorine, and results for the five locations have been pooled (Figure 5.16).

Although *Naegleria* were effectively removed from water in the trunk main at George's Corner (Figure 5.14), they were isolated occasionally from the reticulated water. The majority of isolations (more than 70%) occurred when the free chlorine residual was less than 0.5 $mg.l^{-1}$ (35% of samples).

The origin of the contaminating organisms was not always clear from the sampling. However, some isolations were linked closely (in time and location) to repairs to water mains, suggesting that contamination of the system by soil or groundwater had taken place. This conclusion was sometimes supported by changes in other water quality parameters such as turbidity. Furthermore, the most common amoeba identified in samples from Pt Pirie (and rarely isolated from the trunk main), *Hartmannella vermiformis,* produces cysts and is found in soil as well as freshwater (Page, 1976).

Multiplication of *Naegleria* which survived chlorination in numbers too small to be detected by the sampling at George's Corner was also a possibility.

Pooled results for six locations on the reticulation system of Port Augusta, sampled during the same period, are also shown in Figure 5.16. The frequency distribution of chlorine concentrations was similar to that in Port Pirie, and *Naegleria* were occasionally isolated. Sixty per cent of isolates came from samples collected when the free chlorine residual was less than 0.1 $mg.l^{-1}$ (9% of samples.)

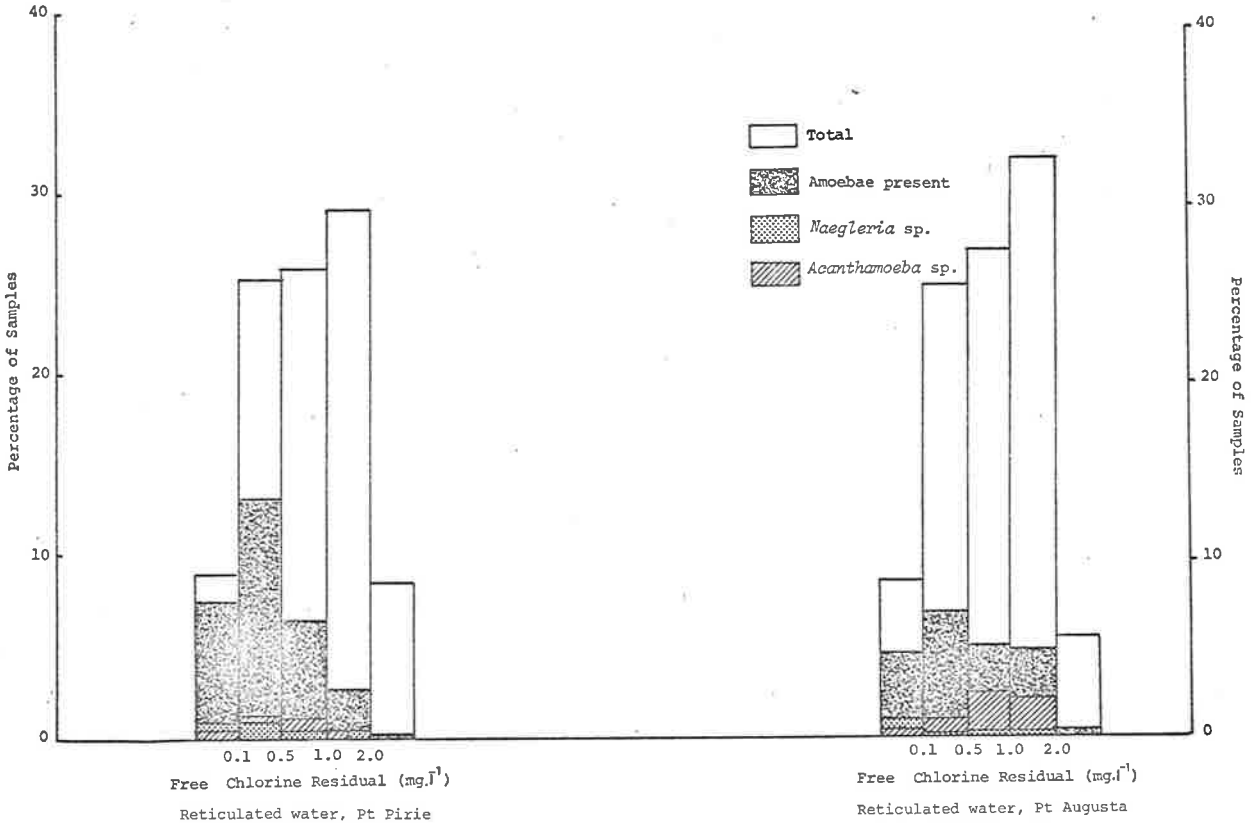


FIGURE 5.16 FREQUENCY OF ISOLATION OF AMOEBAE FROM RETICULATED WATER IN PT PIRIE AND PT AUGUSTA, 1.1.74 - 30.6.79.

Height of each portion of the bar above the base-line represents the percentage of samples in that class.

The few isolations of *Naegleria* at levels of chlorine above 0.5 $mg.l^{-1}$ usually followed isolation of *Naegleria* from the previous sample when very little chlorine was present. In a complex water reticulation system, microorganisms may persist for a time in 'pockets' relatively inaccessible to disinfection. Kadlec *et al.* (1978) showed that part of the water in a swimming pool (with relatively simple water circulation) may be inaccessible to chlorine under some circumstances, although the general level of disinfection is satisfactory. Anderson and Jamieson (1972a) found difficulty in complete removal of *Naegleria* from a swimming pool at chlorine doses which this study suggests should be effective, and concluded that chlorine was not a suitable disinfectant.

The results of sampling in Port Pirie and Port Augusta suggested that initial disinfection may not adequately control *Naegleria* in the reticulated water, and the practice has been to maintain 0.5 $mg.l^{-1}$ free chlorine residual throughout the reticulation system of Port Pirie, Port Augusta and Kadina during the months when infection by *Naegleria* is a risk.

None of the *Naegleria* isolates from reticulated water in these towns was identified as *N. fowleri*. A number of them were serotyped by the Amoebic Research Unit, IMVS, and reported in a frequency distribution of agglutination titres (Jamieson, 1975). They included *N. gruberi* and nonpathogenic isolates which agglutinated with anti-fowleri antisera (presumably *N. lovaniensis*, although other characters have yet to be examined).

Of the nonpathogenic species, *N. gruberi* at least is less susceptible to chlorine (Section 5.5) than *N. fowleri*. Furthermore, many of these isolations were made during the cooler months, when water temperatures were below the lower limit of tolerance of *N. fowleri* trophozoites (Section 4.3). During this period, chlorine doses were reduced to a level adequate to control bacterial contamination.

The frequency of isolation of *Naegleria* at Paskeville No. 1 Reservoir (Figure 5.17), the major source of water for Kadina during this study, was similar to that from Nelshaby Reservoir. *Naegleria* was effectively controlled by disinfection and was rarely isolated from Kadina. *Acanthamoeba*, present in a significant number of samples collected immediately after chlorination, was also less common in the reticulation system.

Three reservoirs serving metropolitan Adelaide were sampled during the same period, as a comparison. Before chlorination at Happy Valley and Kangaroo Creek Reservoirs (Figures 5.18 and 5.19), *Naegleria* and *Acanthamoeba* were often isolated. The frequency of isolation of *Naegleria* after chlorination was very low, but *Acanthamoeba* was occasionally present, even at high free chlorine residuals.

At Hope Valley, South Australia's first water filtration plant commenced operation during the course of this study. The complete water treatment process involves flocculation, settlement and filtration. Although water for the new plant was drawn from a different part of the reservoir, the frequency of isolation of *Naegleria* was very similar to that from the older pump station, and isolation of *Acanthamoeba* a little more frequent (Figures 5.20 and 5.21).

Before commencement of water treatment, over 90% of samples from the trunk main had 1.0 $mg.l^{-1}$ free chlorine residual or greater, and *Acanthamoeba* was present in 21% of these. Since the supply of treated water began it has been possible to maintain lower chlorine doses, which for Adelaide are determined mainly by the bacteriological quality of the water. Between 7.11.77 and 30.6.79 only 6% of samples had as much as 1.0 $mg.l^{-1}$ free chlorine, and *Acanthamoeba* was not isolated.

One or more steps in the water treatment process may be partly responsible for this improvement, and the increased contact time in newly constructed water storage tanks may also have contributed.

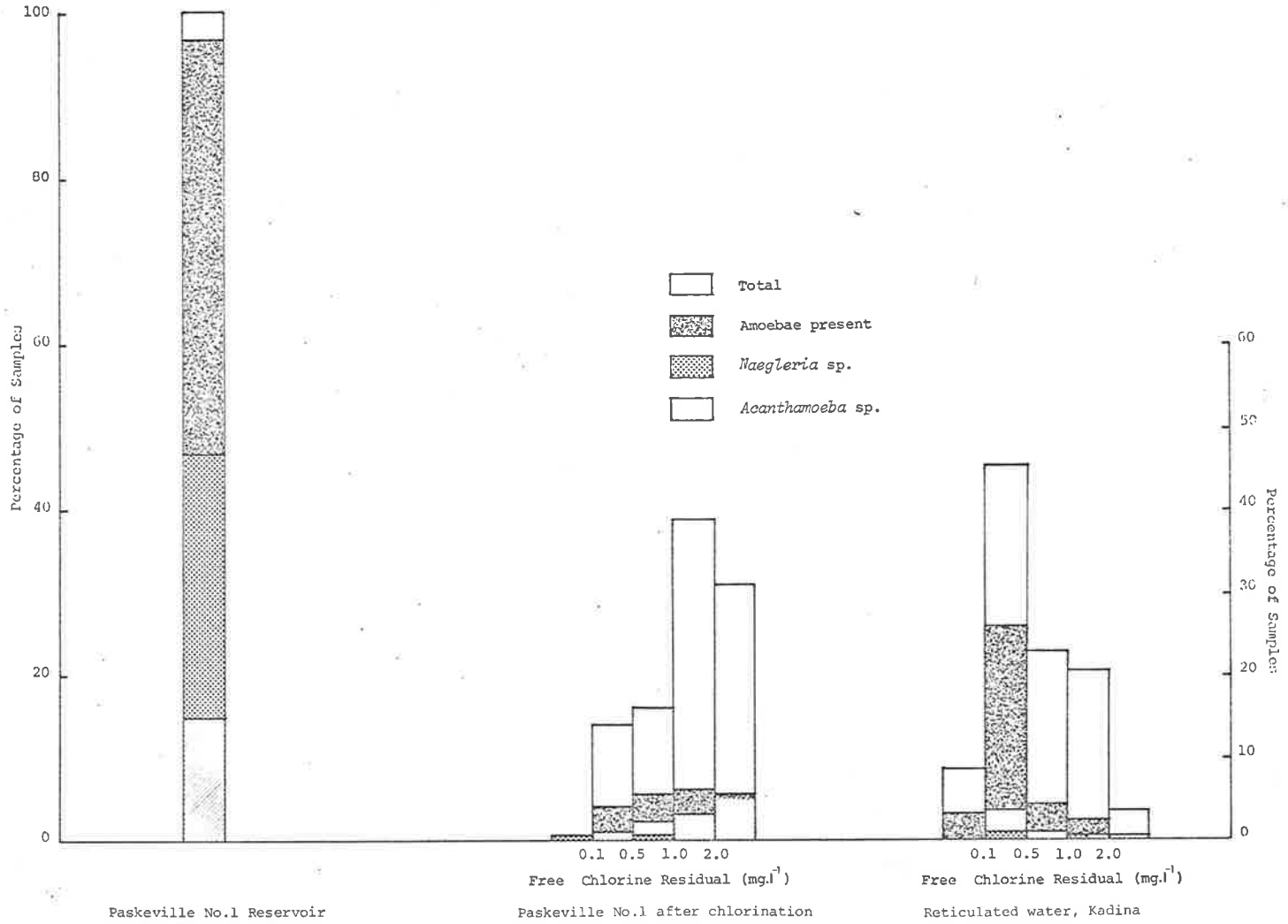


FIGURE 5.17 FREQUENCY OF ISOLATION OF AMOEBAE FROM WATER SUPPLIED TO KADINA, 1.1.74 - 30.6.79

Height of each portion of the bar above the base-line represents the percentage of samples in that class.

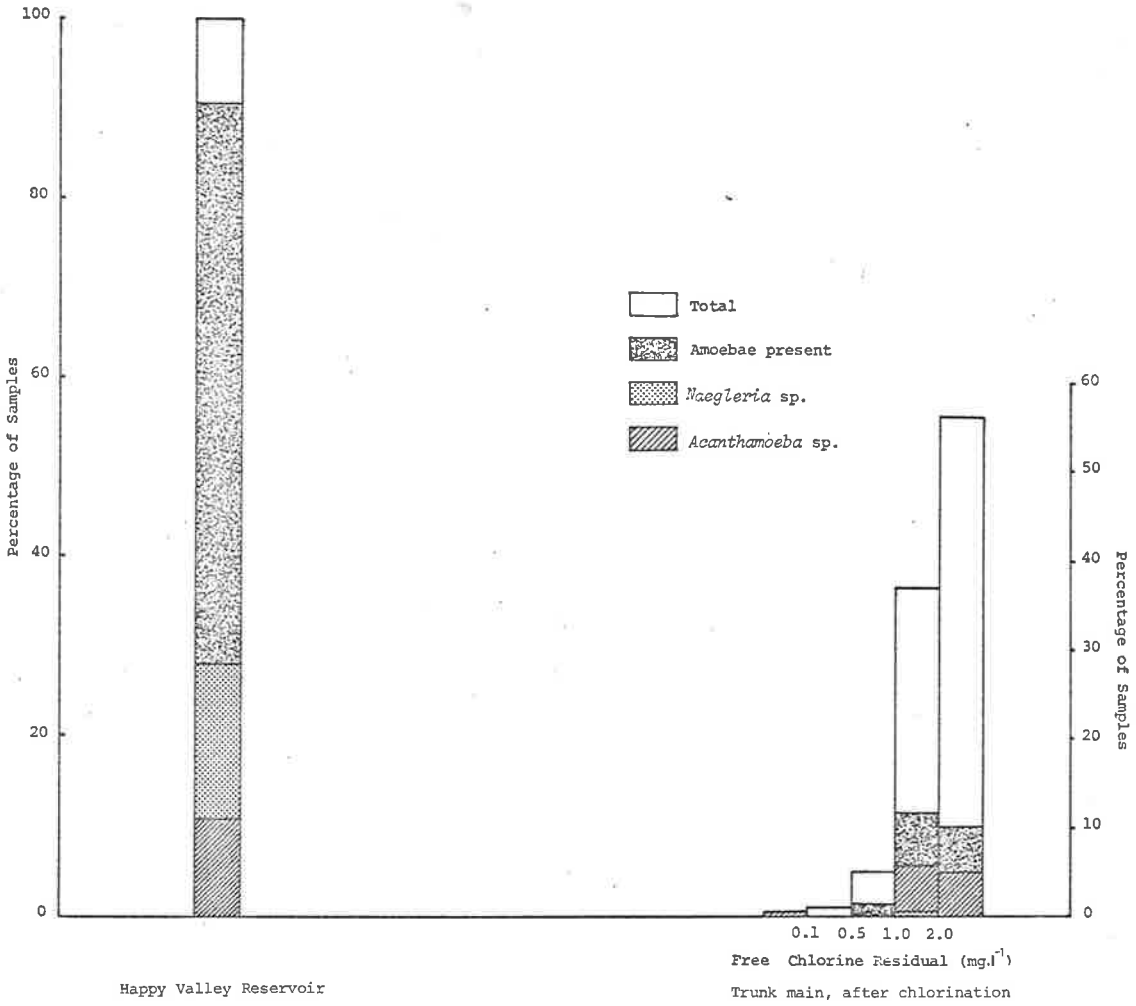


FIGURE 5.18 FREQUENCY OF ISOLATION OF AMOEBAE BEFORE AND AFTER CHLORINATION AT HAPPY VALLEY RESERVOIR, 1.1.74 - 30.6.79

Height of each portion of the bar above the base-line represents the percentage of samples in that class.

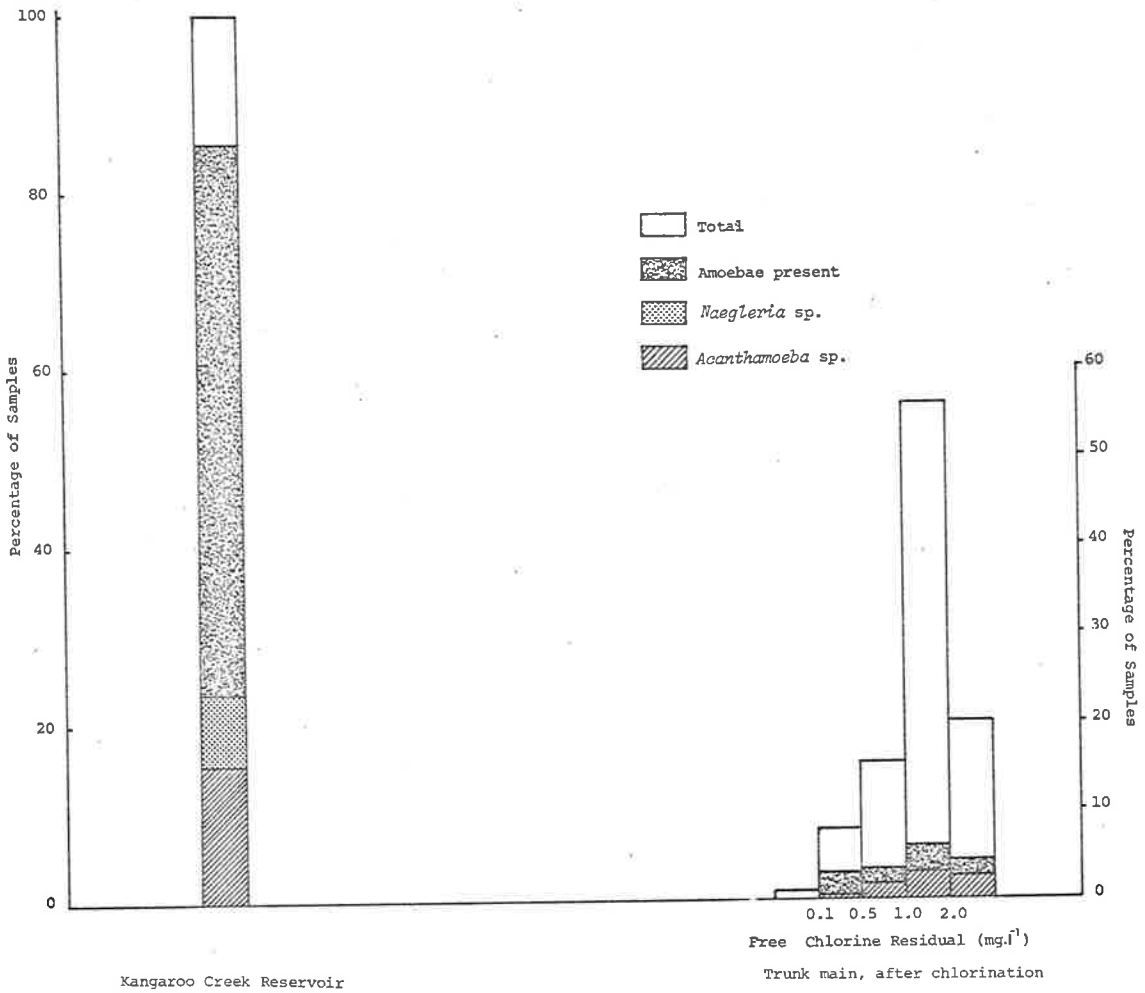


FIGURE 5.19 FREQUENCY OF ISOLATION OF AMOEBAE BEFORE AND AFTER CHLORINATION AT KANGAROO CREEK RESERVOIR, 1.1.74 - 30.6.79

Height of each portion of the bar above the base-line represents the percentage of samples in that class.

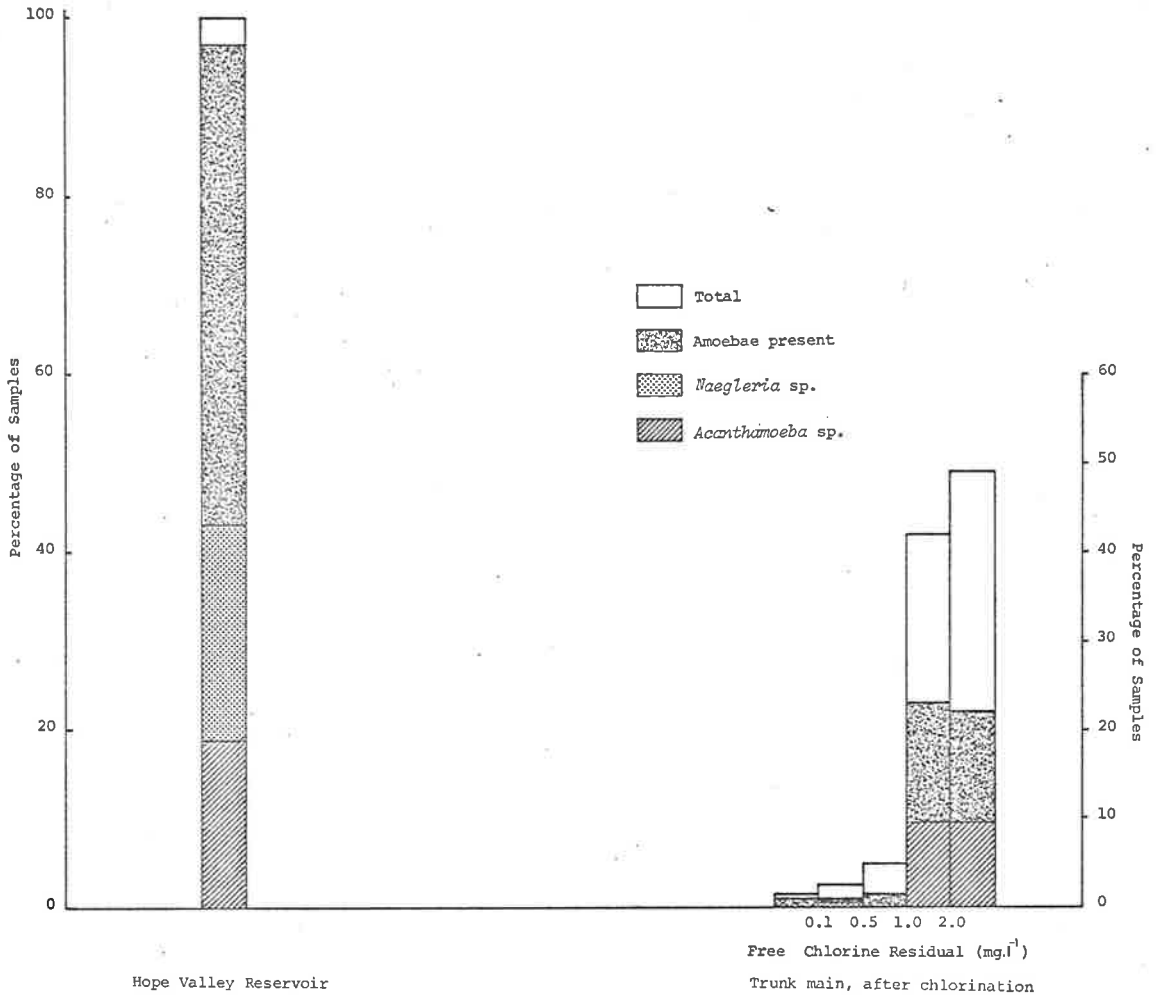


FIGURE 5.20 FREQUENCY OF ISOLATION OF AMOEBAE BEFORE AND AFTER CHLORINATION AT HOPE VALLEY RESERVOIR PRIOR TO COMMENCEMENT OF THE WATER TREATMENT WORKS, 1.1.74 - 6.11.77

Height of each portion of the bar above the base-line represents the percentage of samples in that class.

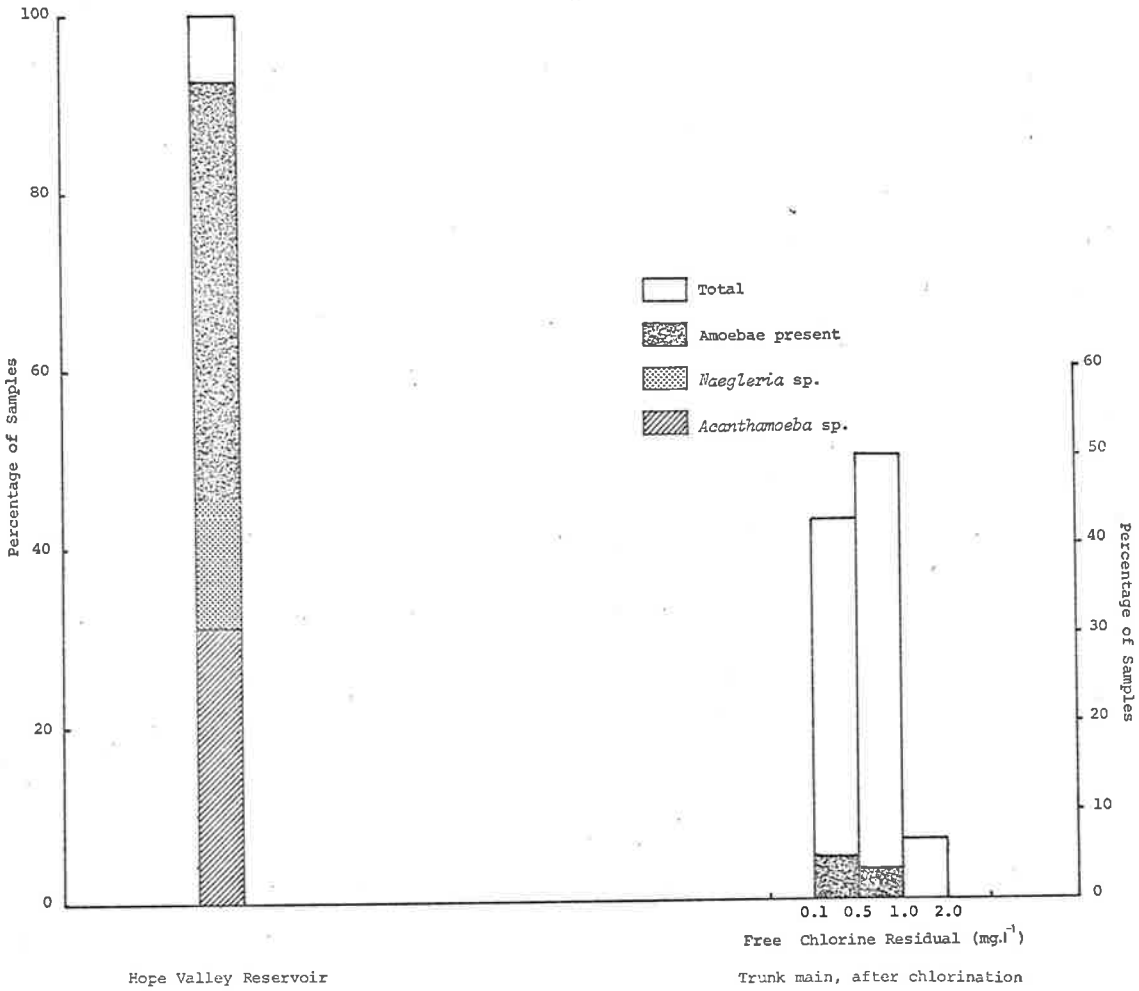


FIGURE 5.21 FREQUENCY OF ISOLATION OF AMOEBAE BEFORE AND AFTER CHLORINATION AT HOPE VALLEY RESERVOIR, FOLLOWING COMMENCEMENT OF THE WATER TREATMENT WORKS, 7.11.77 - 30.6.79

Height of each portion of the bar above the base-line represents the percentage of samples in that class.

The frequency of isolation of *Naegleria* from each of the reservoirs sampled was higher than that of *Acanthamoeba*, while from the two locations on the River Murray, *Acanthamoeba* was more commonly isolated. Since *Naegleria* was isolated from more than 50% of samples collected before chlorination at Nelshaby and Paskeville No. 1 Reservoirs, these reservoirs would be important sources of contamination of the public supply by amoebae of possible clinical importance, should there be a failure of disinfection.

6. CONCLUSIONS

Most of the genera of small, bacteria-feeding amoebae discussed in Page's Illustrated Key were isolated in the sampling study of water sources and public water supplies presented in this thesis (Table 3.1). Indeed, in the Vahlkampfiid genera and *Acanthamoeba*, where more attention was given to specific identification, most of the species recognized in Page's classification were identified.

Page (1976) cautioned that the distribution records cited in the Illustrated Key should not be interpreted to mean that there is a 'narrow geographic limit' to distribution for those species. The observations reported here suggest that most free-living amoebae may have an almost cosmopolitan distribution, albeit with some limitations of habitat.

Naegleria species were among the most frequently isolated amoebae from all water sources sampled. *N. fowleri*, the pathogen responsible for primary amoebic meningoencephalitis, while far less common than other *Naegleria* species and apparently less widely distributed, was clearly a potential contaminant of reticulated water in northern South Australia.

Two new Vahlkampfiid amoebae, assigned to the genera *Tetramitus* and *Vahlkampfia*, have been described here. Both species, while uncommon, must be distinguished with care in any study of the distribution of *Naegleria* species, since their cysts have structural features in common with cysts of that genus. The high temperature tolerance of *Tetramitus ampliporus* suggests that, like the recently described *Naegleria lovaniensis*, it is quite likely to be isolated in association with *N. fowleri*.

It is well established that high water temperatures have an important role in the epidemiology of primary amoebic meningo-encephalitis in several countries, although few comprehensive studies

which included water temperatures have been published. Identification of *Naegleria* species from samples collected as part of epidemiological studies has provided a great deal of qualitative information on the environments in which *N. fowleri* may occur.

Semiquantitative studies (e.g. Wellings *et al.*, 1977) have indicated that numbers of *N. fowleri* are likely to be higher at summer water temperatures or in circumstances where temperature is maintained by thermal springs or industrial discharges.

In this study, examination of a large number of *Naegleria* isolates indicated that the temperature tolerance of non-pathogenic *Naegleria* is extremely variable; the uniformity of the temperature tolerance of *N. fowleri* was confirmed. At three storage reservoirs where *Naegleria* species were common, seasonal changes in the strains isolated were correlated significantly with water temperature. Two of the reservoirs (Nelshaby and Paskeville No. 1) supply water to areas where amoebic meningoencephalitis cases have occurred. While *N. fowleri* was uncommon and was not isolated in some years, the non-pathogenic strains isolated during summer had very high temperature tolerances. However, at Hope Valley Reservoir, part of the water supply for metropolitan Adelaide where no infection by *N. fowleri* has been recorded, *Naegleria* strains with high temperature tolerance were rarely isolated, even during summer.

Thus the geographic and seasonal distribution of *Naegleria* strains tolerant of high temperatures, including the pathogenic species *N. fowleri*, reflects the distribution of known cases of amoebic meningoencephalitis in South Australia.

Experiments were performed to determine the influence of temperature on growth rates of *N. gruberi* and several isolates of *N. fowleri* from human infections and from freshwater. Over most of its temperature range, growth of *N. gruberi* in axenic media or with

bacterial food fitted a straight line of the form

$$k_{24} = a\ (T - T_L)$$

where k_{24} is the growth rate constant, T_L is the lower limit of temperature tolerance, T is the growth temperature, and a is a variable describing other conditions, including availability of nutrients, and depending on the culture history of the *Naegleria* strain. For *N. gruberi*, and probably for *N. fowleri*, since the growth curves of several strains had almost identical shape, T_L appeared to be independent of other growth conditions.

The growth statistics calculated from these experiments (Table 4.3) were used to provide a qualitative explanation for seasonal changes in abundance of *Naegleria* species which are not adequately explained by the upper limits of temperature tolerance.

As water temperature falls during autumn, temperatures which are lethal to trophozoites of *N. fowleri* (16° to 19°C, varying between strains) are reached. In each of the water bodies studied here, temperatures remained below T_L for *N. fowleri* for several months each winter. *N. gruberi*, however, with a T_L close to 7°C, is able to grow throughout the year, albeit slowly at the temperatures measured during winter. When temperatures favourable for growth of *N. fowleri* are reached, presumably only cysts remain. Excystment of *N. fowleri* occurs and its growth rate increases more rapidly than that of *N. gruberi* as temperature continues to rise. Q_{10} values for the interval 20° to 30°C varied from 3.3 to 9.8 for the three *N. fowleri* strains, whereas growth rate of *N. gruberi* increased only 1.7-fold over the same temperature range.

As well as this qualitative explanation, the growth statistics could be used to establish a model of seasonal changes in abundance. As yet there are no published estimates of numbers of *Naegleria* in natural water. Development of quantitative methods which differentiate between

cysts and trophozoites and between *N. fowleri*, *N. gruberi* and, if possible, *N. lovaniensis*, should be a major objective of any future sampling programme.

Mathematical description of the influence of temperature on other physiological processes would be necessary before abundance of *Naegleria* species could be modelled accurately. Q_{10} values and absolute rates for survival of trophozoites and cysts at low temperatures, for encystment and excystment, and for the amoeba-flagellate transformation, should be estimated. It would also be important to determine the range of densities over which log-phase growth can occur under different conditions, and the influence of density of amoebae and the availability of bacterial food on the kinetics of encystment and excystment.

An important benefit of a workable model for abundance of *Naegleria fowleri* in natural water (and a test of its validity) would be prediction of the occurrence of *N. fowleri* in 'significant' numbers in particular water bodies or in a particular year. (In spite of the many epidemiological studies of infections by *N. fowleri*, there has been little published discussion of the significance of different densities of this amoeba, largely because of the lack of quantitative sampling.) There may be, for example, a 'critical length' for the time water temperature is below T_L.

A model might also predict, given bacteriological and temperature data, the likely growth rate of *N. fowleri* in a water tank or swimming pool, should it be contaminated. The growth statistics calculated here have already been used to set seasonal limits to sampling with more confidence.

Assessing the effectiveness of disinfection by chlorine in controlling *Naegleria* species in public water supplies was an important part of this study. Disinfection experiments showed that the effect of

chlorine on cysts of *Naegleria fowleri* was influenced by chemical and physical variables such as pH and temperature in a manner predictable from a knowledge of the chemistry of chlorine in water. In a practical situation, the importance of these variables would depend on the contact time available for the action of chlorine. Under most conditions likely to be encountered in practice in South Australia, survival of several isolates of *N. fowleri* was reduced to below 1 percent by a chlorine dose sufficient to provide 0.5 $mg.l^{-1}$ free chlorine residual after 30 minutes contact with the cysts.

Careful study of the initial kinetics of the action of chlorine on cysts of *N. fowleri* and *N. gruberi* showed that disinfection did not fit the 'pseudo-first order' model often reported for bacteria and viruses. Rather, a distinct lag, which suggested a finite penetration time for entry of chlorine to the cysts, was observed. This experiment also confirmed that *N. gruberi* is less susceptible to chlorine than *N. fowleri*.

While trophozoites of *Acanthamoeba* were very susceptible to chlorine, the cysts of the two strains studied were extremely resistant; 40 to 60 percent survived after one hour with approximately 2 $mg.l^{-1}$ free chlorine remaining in each experiment.

Naegleria species were common in water samples collected from the River Murray and even more common at a number of reservoirs which are sources of public water supply, including those used in the study of seasonal distribution (Section 4.2). *Naegleria* were almost invariably absent from samples collected after chlorination of these supplies, confirming the effectiveness of chlorine in controlling *Naegleria* species. However, the frequency of isolation of *Acanthamoeba* species (presumably present as cysts) was high at some locations, reflecting the resistance to chlorine demonstrated in the disinfection experiments.

Despite adequate control immediately after chlorination, *Naegleria* species were occasionally isolated from reticulated water. These isolates usually occurred at free chlorine residuals well below 0.5 $mg.l^{-1}$, often during the cooler months when chlorine doses used were those adequate to control bacterial contamination, and were identified as nonpathogenic strains, *N. gruberi* and probably *N. lovaniensis*. Possible sources of contamination of reticulated water by *Naegleria* species are discussed in Section 5.7.

Finally, the effectiveness of control of amoebae by comprehensive water treatment (flocculation, settlement, filtration and disinfection) was demonstrated. Improved control, particularly of *Acanthamoeba*, was achieved at a generally lower chlorine concentration following commencement of South Australia's first water filtration plant.

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